ABSTRACT

NIK MOHD AZMI, NIK MOHD FAIZ BIN. Characterization of Marek's Disease Virus-Induced Immunosuppression in Meat Type Chickens using Infectious Laryngotracheitis Challenge Model. (Under the direction of Isabel M. Gimeno/ James S. Guy.)

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by the oncogenic herpesvirus, Marek's disease virus (MDV). In addition to lymphoma, MDV induces immunosuppression (MDV-IS). Pathogenesis of MDV-IS is complex. This PhD work has focused on the study of late-MDV-IS that occurs after MDV establishes latency and during the development of tumors. A series of experiments using commercial meat type chickens bearing maternal antibodies against MDV were conducted to evaluate late-MDV-IS.

The aim of the first objective was to develop a model to study late-MDV-IS under laboratory conditions. The impact of late-MDV-IS was evaluated by assessing the effect of early infection (day of age) with a very virulent plus MDV (vv+MDV) on the efficacy of chicken-embryo-origin (CEO) infectious laryngotracheitis (ILT) vaccine against ILT challenge. Chickens exposed to vv+MDV prior to vaccination with CEO vaccine had similar ($p<0.05$) ILT clinical scores, gross lesions, histopathologic lesion scores, and load of ILTV transcripts in trachea after ILTV challenge, as chickens that were not vaccinated with CEO vaccine. This model was used in all subsequent studies.

The aim of the second objective was to determine whether if currently used vaccine protocols against MD protect against late-MDV-IS. Our results show that none of the currently used vaccine protocols (HVT, HVT+SB-1, or CVI988 administered at day of age, in ovo, or in double vaccination protocols) protected against late-MDV-IS induced by vv+ MDV strains 648A and 686. Experimental vaccine Md5-BACΔMEQ administered subcutaneously at one day of age was the only vaccine protocol that significantly reduced late-MDV-IS induced by vv+MDV strain 686. Differences between Md5-BACΔMEQ and

other MD vaccines could be due to the ability of Md5-BACΔMEQ to compromise reactivation of vv+MDV strain 686 from lymphocytes.

The aim of the third objective was to determine factors (i.e.virus pathotype and host sex) that has an influence on the development of late-MDV-IS. Five strains of MDV representing different pathotypes: virulent (617A, GA), very virulent (Md5) and very virulent plus (648A, 686) were evaluated. Our result showed that only vv+ strains were able to induce late-MDV-IS in this model. In this study both male and female chickens were equally susceptible to MDV-IS induced by vv+MDV 648A.

The aim of the fourth objective was to evaluate if late-MDV-IS can occur in the absence of tumors. A retrospective study using results from 4 animal experiments involving 16 different treatment groups and 487 chickens showed that there was no correlation between development of tumors and late-MDV-IS.

The aim of the fifth objective was to evaluate mechanisms involved in late-MDV-IS by comparing the pathogenesis of three strains derived from vv+MDV 686 (686, 686-BAC, and 686-BACΔMEQ) that differed greatly on their immunosuppressive abilities. Our results showed a clear gradation of virulence and immunosuppressive abilities among the three MDV strains evaluated. The most virulent strain (686) was highly immunosuppressive in the late-MDV-IS model, reduced greatly the humoral immune responses against ILTV and the percentage of MHC-I^+-$CD45^+$ cells in the spleen. Furthermore, it induces the highest frequency of tumors and had the highest level of MDV transcripts and expression of viral miRNA. In the middle, it was the molecular clone 686-BAC that reduced expression of antibodies against ILTV to some degree and expression of MHC-I in splenocytes but it did not induced late-MDV-IS in the ILT model. Furthermore it induced tumors and had high

levels of MDV transcripts and high expression of miRNA, albeit at lower level than strain 686. At the bottom of the spectrum was strain 686-BACΔMEQ that did not induce any immunosuppression or tumors, had no effect on MHC-I expression in splenocytes, and have no, or minimal, viral antigen transcription and/or expression of viral miRNAs.

Characterization of Marek's Disease Virus-Induced Immunosuppression in Meat Type Chickens using Infectious Laryngotracheitis Challenge Model

by
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DEDICATION

I would like to dedicate this thesis to my beloved late grandmother, Nik Wok who is smiling down from above

BIOGRAPHY

Nik M. Faiz was born in Kelantan, Malaysia. He grew in a rural area with agriculture being the main source of income and employment. He always wanted a career where he can make a difference in the lives of people and animals, which is the main reason he went to veterinary school. His interest in poultry started when he was in fourth year of his Doctor of Veterinary Medicine (DVM) program. He was inspired by his undergraduate advisor and his contribution to the poultry industry, he applied as a young lecturer at the Faculty of Veterinary Medicine, Universiti Putra Malaysia, Malaysia. With developing interest in poultry, he furthered his studies in Master of Veterinary Medicine specializing in avian medicine. After completing his Master program, he was offered a scholarship by the Malaysian government to further his studies abroad. He pursued his doctorate degree at the College of Veterinary Medicine, North Carolina State University (NCSU) under supervision of Dr. Isabel Gimeno focusing on Marek's Disease virus (MDV).

Upon completion of his Ph.D from North Carolina State University, Faiz plans on using his knowledge he obtained throughout the years to continue his research interest in poultry diseases in Malaysia. He will be joining Universiti Putra Malaysia to teach future veterinarians, especially in poultry field.

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I have made many friends during my graduate careers. I would like to thank all my friends especially poultry residences for being great officemates and all the fun times together. I also would like to thank all students that has come and go into the lab, you have helped me feel less lonely.

Finally, I want to thank all my family members who continuously giving me moral support and prayer. To my wife, I can’t thank you enough for all the sacrifices you have made all these years. You are my motivation and my strength. Could not have done this without you. And God, thank you for all your blessing and strength given to me.

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LIST OF ABBREVIATIONS

ADOL	Avian Disease and Oncology Laboratory
ATCC	American Tissue Culture Collection
BAC	bacterial artificial chromosome
CD	cluster of differentiation
CEF	chicken embryo fibroblasts
CEO	chicken embryo origin
CKC	chicken kidney cells
CS	clinical sign
DEF	duck embryo fibroblast
dpi	days post infection
ED	days of embryonation
E gene	early gene
FFE	feather follicle epithelium
gB	glycoprotein B
GL	gross lesion
HVT	herpesvirus of turkey
ICP	intercellular protein
IE gene	immediate early
IO	*in ovo*
ILT	Infectious laryngotracheitis
ILTI	Infectious laryngotracheitis index

ILTV	Infectious laryngotracheitis virus
ITPI	intratracheal pathogenicity index
IS rank	immunosuppression rank
L gene	late gene
LATs	latency-associated transcripts
LL	Lymphoid leukosis
MAb	Maternal antibodies
MATSA	Marek’s Tumor Associated Surface Antigen
MD	Marek’s disease
MDV	Marek’s disease virus
MDV-IS	Marek’s disease virus-induced immunosuppression
Meq	MDV EcoRI-Q
MHC	major histocompatibility complex
miRNA	microRNA
mMDV	mild Marek’s Disease Virus
NK cells	natural killer cells
NO	nitric oxide
ORF	open reading frame
PCR	polymerase chain reaction
PFU	plaque forming units
PI	protective index
REV	reticuloendotheliosis virus

SPAFAS	Specific Pathogen Free Avian Supplies
TCO	tissue culture origin
TP	transient paralysis
TXT	treatment
vMDV	virulent Marek’s Disease Virus
vvMDV	very virulent Marek’s Disease Virus
vv+MDV	very virulent plus Marek’s Disease Virus

CHAPTER 1

Introduction and Literature Review

1.1 General introduction

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by the oncogenic herpesvirus, Marek's disease virus (MDV), also known as *Gallid herpesvirus* 2 (GaHV-2). Based on serologic and genomic features, MDV can be divided into three serotypes: oncogenic serotype 1 (GaHV-2) or MDV-1, non-oncogenic serotype 2 (GaHV-3) or MDV-2, and non-oncogenic serotype 3 (MeHV-1) or MDV-3 known also as herpesvirus of turkeys (HVT). MD was first described by Joseph Marek in 1907 as a polyneuritis, affecting older chickens with low or no mortality (1). MD has experienced major changes since then. In the late 1950's, MD experienced the first shift and it became a major problem worldwide. Novel features of MD included development of tumors in nerves and viscera of both old and young chickens and very high mortality. The development of vaccination against MD at the end of 1960's controlled MD very successfully (2, 3). However, outbreaks in vaccinated flocks became common in the 80's leading to the discovery of newly emergent MDV strains able to break vaccine immunity (4). Today, based on the ability to break vaccine immunity, MDV isolates are classified into different pathotypes (virulent (vMDV), very virulent (vvMDV), and very virulent plus (vv+MDV) (4). In addition to lymphoma, highly virulent MDVs (vv and vv+) are able to induce a variety of syndromes unrelated to tumors such as acute transient paralysis (5), lymphodegenerative syndromes (6), panophtalmitis (7, 8), arteriosclerosis (9, 10), and immunosuppression (11-13). Most of these syndromes do not have major relevance for commercial poultry as they are easily controlled by maternal antibodies (MAb) and vaccination. Immunosuppression, however, is poorly understood and its impact in commercial poultry unknown.

Marek's disease virus-induced immunosuppression (MDV-IS) pathogenesis is complex. MDV-IS has been divided into two phases: an early immunosuppression phase associated with early cytolytic infection of the lymphoid organs; and a late immunosuppression phase associated with the establishment of latency and development of tumors (11). Early-MDV-IS is transitory and is controlled by the existence of MAb against MDV or by vaccination (14, 15), thus, it does not have much relevance under commercial conditions. On the other hand, late-MDV-IS is believed to be long-lasting, involves numerous mechanisms, and it is poorly understood. It is likely that late-MDV-IS could occur in commercial poultry but the extent of its impact is unknown since there are no methods to diagnose it. One of the major limitations to study late-MDV-IS is the lack of a model to reproduce it under laboratory conditions.

MDV can dysregulate host immune responses by various mechanism such as downregulation of MHC I (16, 17), dysregulation of MHC II (18), and increased nitric oxide (NO) levels (19). Recently, microRNA have also been associated with MDV-IS (20). In addition, tumor cells can also induced immunosuppression. MD tumors express various antigens that can interfere with immune responses such as chicken fetal antigen and CD30 (21, 22). Inhibition of mitogen induced proliferation of normal spleen cells by MDV lymphoblastoid cells also has been demonstrated (23). Moreover, Li, *et al.* (24) demonstrated that deletion of meq oncogene reduced the immunosuppressive effect of MDV towards humoral immune response against avian influenza and Newcastle disease.

MD is mainly controlled by vaccination (3, 25, 26). MD vaccination protects against lymphomas, transient paralysis (5), lymphodegenerative syndromes (13, 27), panophtalmitis

(8), and arteriosclerosis (28). However, it does not protect against MDV infection or transmission. It is also unknown if vaccination protects against late-MDV-IS. Currently, there are three main MD vaccines used in the field, attenuated serotype 1 MDV (CVI988), a combination of serotypes 2 and 3 (HVT+SB1), and serotype 3 alone (HVT). There are several vaccine strategies to improve vaccine protection such as in ovo vaccination *vs.* one day of age, revaccination *vs* one single vaccination, protective synergism between strains of different serotypes, and the use of adjuvants. Furthermore, recombinant vaccines able to surpass protection provided by CVI988 have been developed. Deletion of the two copies of oncogene meq in vvMDV strain Md5 resulted in a vaccine that lacks the ability to induce tumors and protect very efficiently against early challenge with vv+MDV (29-31). The pitfall of Md5-BACΔMEQ vaccine is that it induced severe early-MDV-IS in chickens lacking MAb and with today's regulations cannot be licensed to be used in commercial poultry.

Besides vaccination, there are other factors that influenced the outcome of MD infection such as MDV pathotype (4, 12), host sex (32, 33), host genetics (34-36), presence of MAb (15, 37-39), and coinfection with other immunosuppressive agents (40-44). Highly pathogenic MDVs (vv and vv+) in addition to breaking vaccine immunity (4), induce more severe neurological lesions (45), replicate faster (46) and induce more severe lymphoid organ atrophy (12). It is unknown if MDV pathotype has an effect on the development of late-MDV-IS. Likewise female chickens are more susceptible to develop MD tumors than males (32, 33) but it is unknown how host sex affects late-MDV-IS. Finally, genetic resistance to MD has been amply studied (34-36). In particular the role of B-haplotype on the

development of tumors (35, 36, 47-49), transient paralysis (50), and vaccine protection (51-53) has been well documented but it is unknown if it has an effect on late-MDV-IS.

The aim of this work was to evaluate if late-MDV-IS has an impact in commercial poultry and if so to understand the factors that influence it. The specific objectives of this study were: 1) to develop a model to study late-MDV-IS under laboratory conditions (ILT-model) in commercial meat type chickens; 2) to evaluate whether currently used vaccine protocols against MD protect against late-MDV-IS; 3) to evaluate whether viral pathotype and host sex affect the development of late-MDV-IS; 4) to evaluate whether tumors are necessary for the development of late-MDV-IS; and 5) to evaluate mechanisms involved in late-MDV-IS.

Literature Review

1.2 Marek's Disease

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by *Gallid herpesvirus-2* or commonly known as Marek's disease virus (MDV). MD affect both commercial and backyard chickens and is a great concern to the poultry industry as it can cause remarkable economic losses without proper control. It was estimated that mortality and carcass condemnation losses due to MD is in the range of US$1-2 billion annually worldwide (54).

MD was first described by Joseph Marek in 1907 (1) as fowl paralysis (polyneuritis) caused by inflammation of the peripheral nerves. Several years later, the disease was associated with the development of tumors in visceral organs and it was named '*neurolymphomatosis gallinarum*' (55). Until 1960, it was difficult to distinguish MD from other neoplastic diseases caused by retroviruses (such as lymphoid leukosis or LL) as there were no diagnostic tests to differentiate them. However, in the early 1960's MD was identified as a separate entity from LL (56-58).

As the poultry industry rapidly intensified in the late 1950's, acute MD characterized by tumors in multiple viscera, muscles and skin became more prevalent (59). By the early 1960's, MD became one of the major causes of condemnation in broiler carcasses (60). As a consequence of the major economic losses, much research effort and funds were directed into finding a method of control against this devastating disease, including breeding genetically resistant chickens against MD (61). In the late 1960s and early 1970s, MD vaccine was introduced worldwide and the incidence of clinical diseases declined dramatically (2, 25, 62).

In the USA, from 1970 to 1982, condemnation of young chickens was greatly reduced (1.57% to 0.08%, 94.9% reduction) (63). However, the additional cost of vaccination was introduced. MD vaccines are more expensive than other live vaccines. They are cell associated and they need to be maintained in liquid nitrogen (-196°C). Furthermore special care in handling is required and they are administered at the hatchery. In the USA, vaccination cost were estimated at 44.4 million USD in 1985 (63) and 169 million USD in 2004 (54).

Vaccination was efficacious against the development of the disease (2, 25, 26). However vaccines do not protect against infection and transmission and that might have contributed to the evolution of MDV towards more virulence (4, 6). As MDV has increased in virulence, it has acquired the ability to induce a variety of non-neoplastic syndromes (5, 6, 8, 9). One of those syndromes, MDV-IS, is poorly studied, might have great impact in commercial poultry, and it is the topic of this study.

1.2.1 Disease history

The disease was first described in 1907 by Joseph Marek, a Hungarian veterinary pathologist in his publication entitled “Multiple Nervenentzündung (Polyneuritis) bei Hühnern” (1). The paper describes a disease in four adult roosters as “polyneuritis” based on the findings of paralysis of legs and wings with thickening of sacral plexuses and spinal roots due to mononuclear cells infiltration. The publication did not stimulate interest for over a decade until later findings by Papenheimer et al. showed that the disease was not limited to the nervous system alone but rather a lymphoproliferative process affecting peripheral nerves and visceral organs (55, 64). Based on the findings, they renamed the disease as

neurolymphomatosis gallinarum. The findings that this disease could also induce lesions in viscera complicated its diagnosis as it shares many similarities with another disease, lymphoid leukosis (LL), that had been described by Ellermann (65). The confusion between MD and LL continued for decades until a clear classification of the two diseases was presented at the World Veterinary Poultry Association by Biggs and Campbell (56, 66). As a result, the disease originally described as "polyneuritis" by Marek (1) and "neurolymphomatosis gallinarum" by Pappenheimer, *et al.* (55) was named "Marek's Disease". The disease described by Ellermann (65) was named lymphoid leukosis.

The first experimental transmission of MDV was successfully done in 1960s (67, 68). Later, MDV was shown to be highly cell associated and the cytopathic effect was typical of herpesviruses (69, 70). Furthermore, viral antigens were demonstrated in the feather follicle epithelium of infected chickens which explains the contagious nature of MDV (71-73). Finally, the etiological agent of MD as a herpesvirus was confirmed following vaccine trials using attenuated MDV-1 strain and HVT (2, 3, 25, 26).

MD has changed greatly through the years. As the poultry population expanded between 1950s and 1960s, MD became devastating to the poultry industry around the world. In the mid-1950s, an acute form was reported in broiler chickens characterized by multiple organ tumors that had become a major cause of condemnation of commercial broilers (59, 60). This form of MD was shown to be more severe than the one described earlier and it was named as acute MD to differentiate it from the previously described classical form (74). By mid 1960s, this form was described as an epizootic disease in USA and most major poultry producing countries. The MD outbreaks that occurred in 1960s wiped out large populations

of poultry flocks across the globe (4). From 1960s to 1970s, trait selection towards MD-resistant chickens was a common control procedure despite the potential reduction in other traits. In the late 1960s and early 1970s, MD was successfully controlled by the introduction of the first vaccines, and genetic selection for MD resistant traits became an adjunct to vaccination (2, 25, 62, 75). The vaccine was a huge success for the poultry industry and also was the first vaccine against cancer ever developed.

However in the USA, within 10 years of the introduction of the first vaccine, there were reports of outbreaks in vaccinated flocks (6, 76). Those outbreaks were induced by MDV strains of greater virulence than the first isolates (77). In response to increasing outbreaks in vaccinated flocks in the 1980s, new vaccine strategies based on serotype 2 MDV were introduced (78, 79). Protective synergism was demonstrated by combination of HVT vaccine with serotype 2 MDV (26, 78, 80). The strategy was proven effective until new pathogenic strains emerged and more virulent pathotypes of MDV were isolated in vaccinated flocks. In the USA, serotype 1 MDV vaccine CVI988 was introduced in 1992 to control newly emergent vv+MDV. CVI988 had been used in Europe and in other countries since 1972 and it continue to be in use (25).

Today, MD differs greatly from the first descriptions (1, 55, 59, 81). MDV not only induces lymphoma in nerves and viscera but also a variety of syndromes such as acute transient paralysis (5), lymphodegenerative syndromes (6), panophtalmitis (7, 8), arteriosclerosis (9, 10), and immunosuppression (11-13).

1.2.2 Epidemiology

MDV is transmitted by the airborne route. The transmission of MDV is via contaminated feather dander either by direct or indirect contact with chickens (82). Epithelial cells in the feather follicle epithelium (FFE) support fully productive infection and are the main source of transmission. The keratinized epithelial cells containing fully infectious viral particles contaminate the environment and infect susceptible chickens (72). Virus shedding begins about two weeks MDV post infection (83). Once an animal is infected, they can shed the virus for the rest of their lifespan (84).

Chickens are the most important host for MDV however other poultry species (quails, turkeys and pheasants) are susceptible to infection and can develop disease (85). MDV is ubiquitous and is found in any poultry producing countries worldwide. However, the true incidence of MD is difficult to determine due to several reasons. Morrow and Fehler (54) identified several reasons behind this difficulty: 1) MD is not a notifiable disease; 2) low level losses of MD from MD vaccination are generally accepted and treated as normal, since it is known that efficiency of MD vaccines is not 100%; 3) occurrence of MD is often linked to financial claims between rearing companies and hatcheries or hatcheries and vaccine manufacturers and often such cases are not made public; 4) Since prevention of the disease requires optimal hygiene and management, besides other measures, many MD cases are not reported in order to avoid damaging the reputation of the company concerned. Infection does not necessarily result in clinical disease, especially in vaccinated and genetically resistant

chickens. Furthermore, the number of unreported cases of MD is very high. Hence, the incidence of infection is definitely higher than the incidence of disease (85).

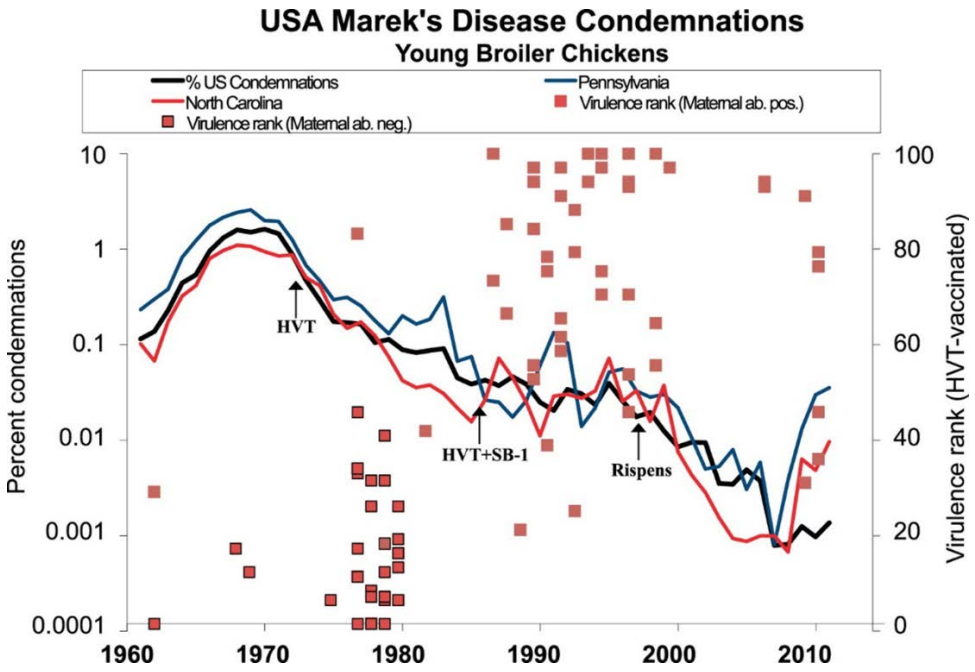

Figure 1.1 U.S. Marek's disease condemnations in young broiler chickens versus virulence rank of pathotyped field strains in HVT-vaccinated birds. Data are also included from the states of Pennsylvania and North Carolina to highlight recent increases in condemnation rates. Arrow indicates the approximate years the specified vaccines were introduced in the United States for all types of poultry. (86)

In the USA, MD prevalence can be illustrated using carcass condemnation data collected since 1961 at processing plants. Several studies using condemnation data have been published previously (63, 86-88). These studies has shown that MD incidence has continued to decrease since 1960s (Figure 1.1). In addition, these data also shown the fluctuation of MD incidence within a year (88). The rapid fluctuation suggested that seasonal weather and management practices plays a key role in the ecological dynamic of MD. Season changes in

ventilation, house cleanout and other aspects related to hygiene may affect the incidence of MD (88). It has been speculated that the non-sterilizing nature of MD vaccine could have driven evolution of MDV towards more virulence (89, 90).

1.3 Marek's Disease virus

1.3.1 Morphology and classification

The causative agent is a cell associated α-herpesvirus known as Marek's Disease virus (MDV). MDV has a linear double stranded DNA genome surrounded by 100nm diameter icosahedral protein capsid core consisting of 162 hollow capsomers (70). The virion are commonly seen in the nucleus and less often in the cytoplasm and extracellular spaces. Nucleocapsids and enveloped particles may be seen in thin sections of infected cell cultures. Non enveloped particles are less commonly seen, amorphous structures of 273-400 nm in size can be observed in thin sections of negatively stained lysed feather follicle epithelium (72).

Herpesviruses are divided into three families: *Herpesviridae, Alloherpesviridae* and *Malacoherpesviridae*. There are three subfamilies in the *Herpesviridae* family namely *Alphaherpesviridae, Betaherpesviridae* and *Gammaherpesviridae*. The *Alphaherpesviridae* consist of four genera: *Simplexvirus, Varicellovirus, Mardivirus* and *Iltovirus*. As per recent classification by the International Committee of Taxonomy of Viruses (ICTV), MDV is included in the genus *Mardivirus*. Members of the genus *Mardivirus* are grouped in three species: *Gallid herpesvirus 2* (GaHV-2), *Gallid herpesvirus 3* (GaHV-3) and *Meleagridis herpesvirus 1* (MeHV-1) (Table 1.1). The species correspond to the MDV serotypes reported in a previous classification based on the variation in antigenic determinants (91, 92).

Serotype 1 corresponds to GaHV-2, serotype 2 to GaHV-3 and serotype 3 to MeHV-1. Serotype 1 (MDV-1) that infects chickens are the only one linked to oncogenicity. Serotype 2 (MDV-2) isolated from chickens and serotype 3 (HVT) isolated from turkeys do not have oncogenic potential. All three serotypes of MDV have been used as vaccines (3, 25, 79).

Table 1.1: Classification of MDV

Species	Serotype	Natural host	Oncogenic potential	Available in vaccine form
Gallid herpesvirus 2 (GaHV-2)	MDV-1	Chicken	Yes	Yes
Gallid herpesvirus 3 (GaHV-3)	MDV-2	Chicken	No	Yes
Meleagridis herpesvirus 1 (MeHV-1).	HVT	Turkey	No	Yes

Shifts in virulence among MDV serotype 1 strains have been recognized since 1970s following unexplained increases in MD losses in vaccinated flocks (76). Classification of serotype 1 MDV in pathotypes using the Avian Disease and Oncology Laboratory (ADOL) assay is the gold standard method for classifying MDV serotype 1 strains (4). The ADOL pathotyping assay was designed to determine the ability of a MDV isolate to break vaccine immunity conferred by HVT and HVT+SB-1 in comparison with prototype strains. The assay is performed in 15x7 maternal antibody positive (dams vaccinated with MD vaccines of all three serotypes) chickens but it could be done in commercial SPF chickens (93). Chickens are vaccinated at hatch, challenged at 5 days of age and evaluated for tumor

development 8 weeks following challenge. Details of pathotyping assay is presented in Figure 1.2.

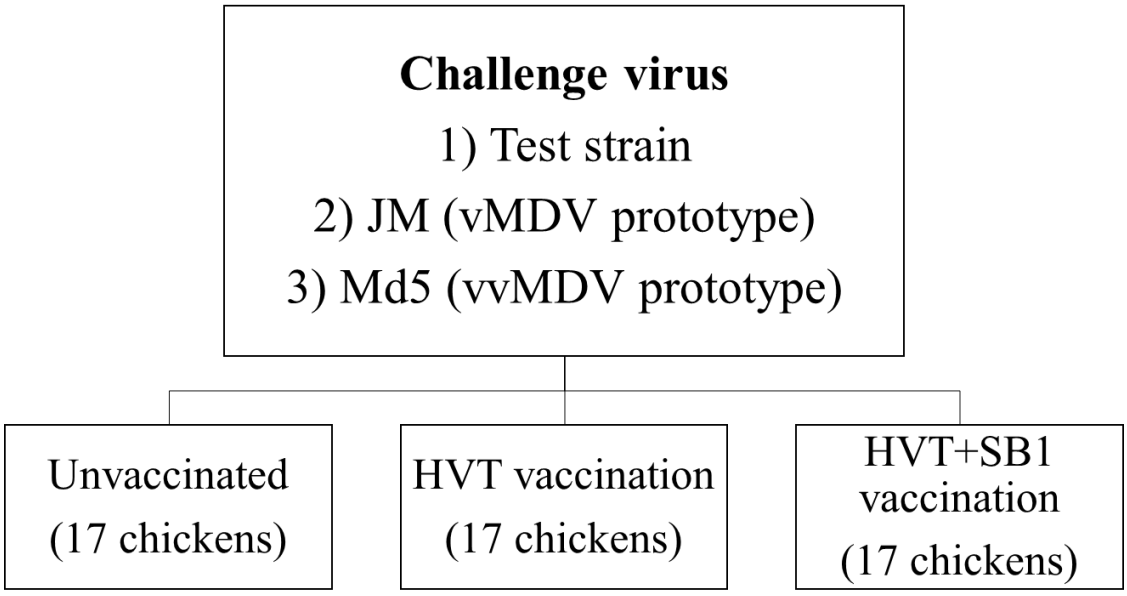

Figure 1.2 ADOL Pathotyping Assay (4)

Table 1.2: ADOL pathotype classification of MDV-1	
Mild (m) MDV	Responses in unvaccinated group are less than JM/102 challenge group
Virulent (v) MDV	Responses in HVT-vaccinated chickens does not differ from JM/102 challenge group
Very virulent (vv) MDV	Responses in HVT-vaccinated chickens exceed JM/102 challenge group but does not differ from HVT-SBI vaccinated Md5 challenge group
Very virulent plus (vv+) MDV	Response in HVT-SB1 vaccinated chickens exceed Md5 challenge group

Based on the assay, serotype 1 MDV can be divided into mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+) MDV-1 (Table 1.2).

1.3.2 MDV genome

All members of the *Herpesviridae* family are double-stranded DNA viruses that range in size from 108-230 kbp (94). Within family *Herpesviridae*, there are six classes (A through F) and only class D and E are in the *Alphaherpesvirinae* subfamily. MDV- 1, MDV-2, and HVT are all in Class E. Class E *Alphaherpesvirinae* genome structure comprise of two unique sequences called unique long (U_L) and unique short (U_S)—each of which is flanked by inverted internal (IR_L, IR_S) repeats and terminal repeats (TR_L, TR_S) (95) (Figure 1.3).

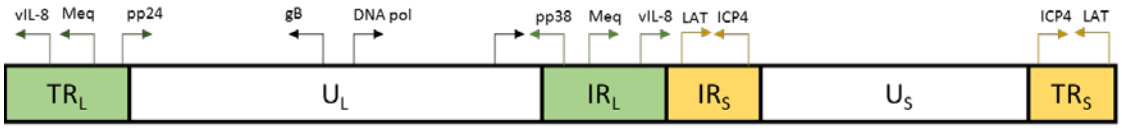

Figure 1.3 MDV-1 genome structure with important transcripts. Transcript locations are drawn in their respective repeat or unique regions in proper order and not drawn to scale. (Modified from Osterrieder and Vautherot (95))

MDV genes can be divided into two categories, genes homologous with alpha herpesviruses and genes unique to MDV. There are also genes that are unique to each MDV serotype. The open reading frames (ORF) of herpesviruses are categorized as either immediate early (IE, α), early (E, β), or late (L, γ) genes. The first genes expressed are the IE genes. Four IE genes have been identified namely intercellular protein (ICP) 4, ICP0, ICP22 and ICP27. ICP4 protein has been shown to be a transactivator based on transfection of MD cell line MSB-1 with the short form ICP4, resulted in increased transcription of endogenous

ICP4, pp38 and pp24 genes (96, 97). In addition, ICP4 protein could transactivate the LTR Rous sarcoma virus at low level (98). Transcription of ICP4 may require the presence of tegument protein called *VP16* (*α-TIF*, α-trans-inducing factor) carried in with the invading virion which activates transcription of α-genes (99).

Early or beta-genes are expressed before replication of viral genome, as these proteins are involved in the replication process (ie. a dsDNA binding protein, or a DNA_{pol}). Many β-genes contain nuclear localization signal motifs that can bind with transport proteins and carry them back to the nucleus. Nuclear β proteins promote the transcription of γ1 (late) and γ2 (true late) genes which are activated at the beginning of viral DNA replication. Late or gamma-genes generally repress transcription of α- and β- genes and codes virion structural proteins such as nucleocapsid, envelope spike proteins, and VP16/other tegument proteins. These γ-gene products are transported to a different locations within the cell, to be assembled into mature virions.

Several genes has been identified that are unique to for *Mardivirus* genus of the family *Alphaherpesviridae*; some are present in only serotype 1 MDV. MDV-1 has ORFs for *Meq* (MDV EcoRI-Q) and interleukin-8 (*IL8*), which is absent in MDV-2 or HVT. Phosphorylated polypeptide *pp38* is also present in MDV-1 genome but absent in MDV-2 and truncated in HVT (100). *Meq*, *vIL-8*, and *pp38* that are only present in serotype-1 MDV isolates have been considered to play a role in oncogenesis (31, 101-105).

Meq is a 339- amino acid protein characterized by a N-terminal bZIP (basic leucine zipper) domain similar to the *c-Jun* oncogene and a proline-rich transactivation domain homologous to the Wilms' tumor suppressor gene (*WT-1*) near its C-terminus (106). The

bZIP domain is the site of *Meq* dimerization resulting in homodimers (107). The bZIP domain is also involved in the association of Meq with other proteins in the *Jun/Fos* family such as *c-Jun* (the primary dimerization partner), *JunB, ATF2*, or *c-Fos* (108). The Meq protein is consistently expressed in the nucleus, nucleoli, and Cajal bodies of lymphoma cells and tumor cell lines (104, 109, 110). Evidence suggests Meq as the principal oncogene responsible for transformation. Xie et al (111) has shown that Meq is required for maintenance of transformed status of MDV-transformed lymphoblastoid cell lines (MDCC-MSB1). Liu et al (104) provided evidence that over expression of Meq resulted in the transformation of rodent fibroblast cell line. Strong evidence that meq is the principal oncogene comes from a study by Lupiani et al (31) where deletion of both copies of Meq gene resulted in full abrogation of the transforming ability of vvMDV strain rMd5. In addition to the transforming and mitogenic properties of Meq, Meq infected cells are highly resistant to apoptosis (104). Meq was also shown to bind to *p53* (112), inhibiting downstream *p53*-mediated apoptotic and cell cycle arrest signaling pathways. The combined effects of serum-independent growth, dysregulation of cell cycle and anti-apoptosis effect resulted in the transformation.

Viral *Il-8* (*vIL-8*) was originally identified as spliced Meq variant and was named *vIL-8* because of its homology to chicken *IL-8* (*chIL-8*) (101, 113-115). The gene consists of 3 exons and is expressed late during cytolytic infection. Recently, *chIL-8* was found to be downregulated in response to MDV infection (116) while other expression analyses show that *vIL-8* is expressed freely (105, 117). Since *vIL-8* functions as a chemoattractant for other lymphocytes, production of vIL-8 by infected B and T cells will attract T cells to aid the

switch from B to T cells during lytic infection (118). Previous studies suggest that *vIL-8* is involved in lymphoma formation via activation of T-lymphocytes (105). Additionally, it was shown that vIL8 is involved in early cytolytic infection but dispensable for the establishment of latency (119).

MDV phosphorylated protein complex, often referred to as pp38/pp24 is coded by 2 genes located at opposite ends of the U_L region (120). Phosphorylated polypeptide pp38 is a lytic gene located in the IR_L region of MDV-1. It is expressed with early kinetics in the infection cycle, along with its inverted repeat, pp24, in the TR_L region. pp38 was originally identified as an antigen expressed on tumor cells and lymphoblastoid cell lines suggesting its role in tumorigenesis (102). However, further studies showed that the expression was limited to small portions of lymphoma cells (110). Deletion of pp38 in Md5 strains resulted in reduction in early lytic replication but did not eliminate oncogenicity (121). Further study by Gimeno et al (122) showed that pp38 is required to establish cytolytic infection in B cells but not in FFE, and to maintain the transformed status of lymphocytes by preventing apoptosis. Recently, Schat, *et al.* (123) reported the presence of two splice variants for pp38 and full length pp38 is essential for the early viral replication. They had also demonstrated that pp38 causes a deficit in ATP availability which could lead to the induction of apoptosis (124).

Another gene unique to MDV are latency-associated transcripts (*LAT*s). These genes are antisense to *ICP4* and highly associated with establishment of latency. Expression of *LATs* leads to transcription of protein that can bind to *ICP4* transcripts, and reducing *ICP4* expression. Studies show that null-*LAT* virus reconstituted from BACs is unable to form tumors or suppress the immune system in the RB-1B strain of MDV-1 (125).

Recently, oncogenesis of MDV-1 have been connected with MDV1-specific miRNA clusters. miRNAs are short (~19-24 nucleotides) RNAs that are transcribed from noncoding genomic regions and function as post-transcriptional regulators (126). There are three miRNA clusters located in the repeat regions. Clusters 1 and 2 flank the Meq ORF, while cluster 3 is at the 5' end of the LAT ORF. Morgan *et al.* reported differential expression of MDV-1 microRNA in MDVs, with higher expression of mdv1-miR-M4 and mdv1-miR-M2 in highly virulent MDVs than in less virulent MDVs (127). mdv1-miR-M4 is a well characterized miRNA of MDV-1 that shares the same seed sequence with chicken gga-miR-155 and also with Kaposi's sarcoma herpesvirus microRNA kshv-miR-K12-11 (127-129). mdv1-miR-M2-3p is another miRNA in the Meq miRNA cluster located next to mdv1-miR-M4-5p. Previous study by Goher *et al.* (130) identifies mdv1-miR-M2-3p binding site to be cluster of differentiation 86 (CD86) and cluster of differentiation 80 (CD80). They hypothesized that mdv1-miR-M2-3p might be involved in T cell activation and responsible to the tumor resistance on chickens vaccinated with HVT (130).

1.4 Pathogenesis

MDV spreads by contact with contaminated environment or with infected birds. Susceptible chickens are infected with MDV by inhalation of infected dander or dust (73). After the virus is introduced into a chicken flock, infection will spread from bird to bird regardless of vaccination status or genetic resistance. An early study has shown that virus excretion begins about 2 weeks post MDV infection (83) and will continue indefinitely (84).

The portal of entry is thorough the lung although the actual virus uptake location and cellular mechanism involved in the uptake have not yet identified. Upon entry of the virus, Calnek postulated four phases of infection in the pathogenesis of MD (131). The phases are known as Cornell model and the sequential events are: 1) early cytolytic phase, 2) latent phase, 3) late cytolytic phase and 4) transformation phase (Figure 1.4). The division are arbitrary with phase 2 - 4 could exist in different cells in the same birds, however, the first two stage can be clearly distinguished.

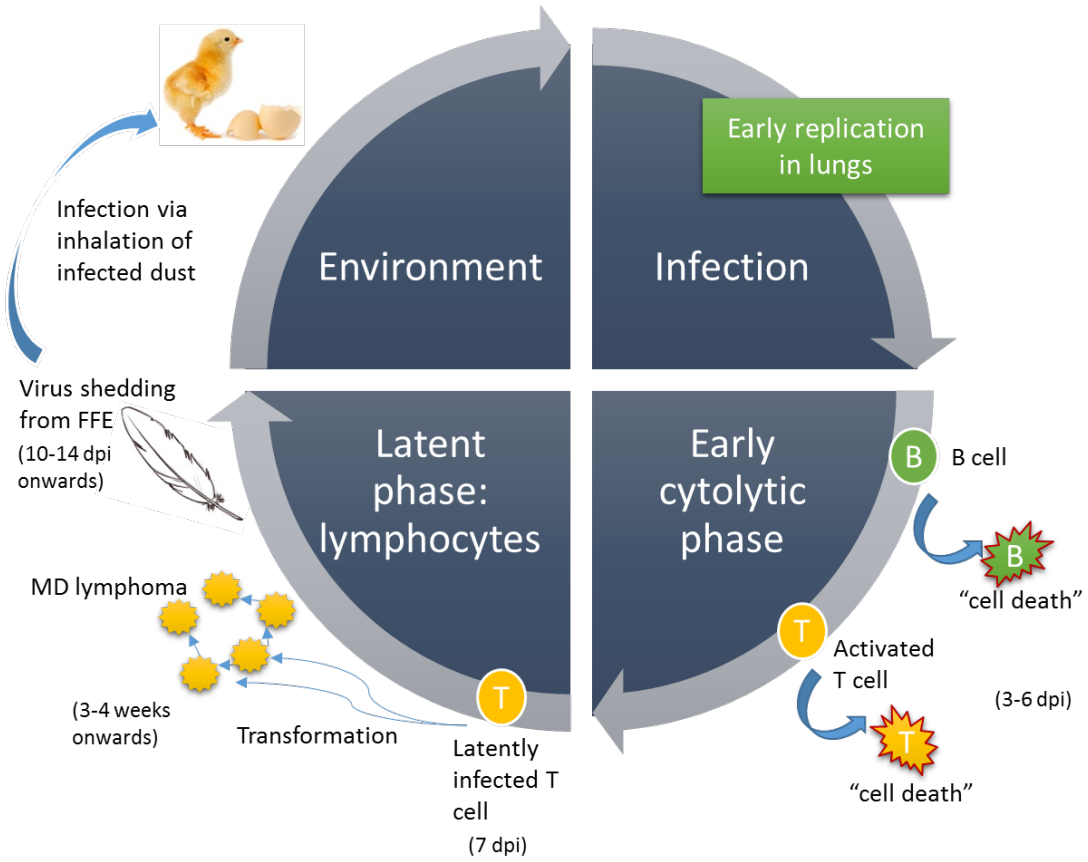

Figure 1.4 Stages in the cycle of MD pathogenesis in susceptible chicken (Modified from Schat and Nair (85))

1.4.1 Early cytolytic phase

Upon entry into the host via respiratory tract, the cell free virus reaches the lymphoid organs within 24-36 hours. Phagocytic cells might be responsible in the transportation of MDV to the lymphoid organs. Shortly after infection, cytolytic infection can be detected in the thymus, spleen and bursa, peaking between 2 and 6 days. The main target cells of early cytolytic phase are B lymphocytes (132). However, a small percentage of $CD4^+$ and $CD8^+$ cells expressing TCRαβ can become cytolytically infected during this stage (133, 134). As a result, transient atrophy of bursa and thymus can be observed at this stage. Depending on the virulence of the MDV, birds may recover between 8 and 14 days post infection or the atrophy could be permanent (12, 13, 135).

Change in the proinflammatory cytokines expression occur in splenocytes during cytolytic phase. Cytokines IFNγ mRNA, IL-1β, IL-8, IL-6, IL18 and inducible NO synthase (iNOs) are upregulated (19, 116, 136, 137). The pathotype of infecting virus and genotype of the host affecting the level of upregulations of these cytokines (138, 139). In the spleen, B lymphocytes are surrounding the ellipsoidal-associated reticular cells (140, 141) and this might explain why B cells are the primary target for acute cytolytic infection. However, removal of spleen prior to MDV infection does not prevent visceral lymphoma development (142) which implies that spleen is important but not essential for early pathogenesis and subsequent event. In addition, it has been shown that genetically resistant line 6 chickens have significantly fewer infected B lymphocytes in the spleen compared to susceptible line 7 chickens (143). Studies by Baigent et al. (133, 134) showed that line 7 chickens cells have more pp38 expression than line 6 chickens although there are more B cells in line 6. $pp38^+$ B

cells in line 7 also surrounded by TCR$\alpha\beta^+$, $CD4^+$ and $CD8^+$ cells which provide optimal condition for virus transfer from B to T cells. This suggested that MDV spread more efficiently in line 7 chickens than line 6 chickens.

It is well accepted that resting T cells are refractory to infection but infected B cells induce activation of T cells and render them susceptible to cytolytic infection during this early phase (144). IFNγ is activated 3-4 days post MDV infection and this may upregulate the expression of IL-8 receptor on activated T cells, which vIL-8 can attract activated T cells to lytically infected B cells and aid in the transfer of virus (101, 145). Study using vIL-8 deleted mutant showed reduction in virus replication during early cytolytic infection as well as tumor formation (101, 119, 146). Moreover, it has been shown that pp38 is essential for early cytolytic infection (122).

There are several factors that could affect the pathogenesis during early cytolytic phase. Vaccination prior to MDV infection and presence of maternal antibody in the host will reduce the cytolytic infection (15, 37, 131, 147). Exposure at one day old leads to longer cytolytic infection compared to exposure at 2 or 7 weeks of age (148). Furthermore, MDV pathotype affects the outcome of early cytolytic infection. MDV strains of the vv and vv+ pathotypes can cause severe lymphoid atrophy and early mortality syndrome compared to less virulent strains (6, 12).

1.4.2 Latent phase

The switch from cytolytic infection to latency occur around 6-7 days post MDV infection. At that time, evidence of cytolytic infection in the lymphoid organs are minimal

and tumors are not yet detectable. Competence of cell mediated immunity (148) and virulence of MDV strains (149) plays a crucial role in the onset of latency.

The targets for latent infection are the activated T cells, mainly CD4+ cells although CD8+ T cells and B cells can also be involved (132, 144, 150). Schat, *et al.* (151) had demonstrated the importance of T cells in latency by showing that latent infection and tumors can occur in embryonically bursectomized chickens. Several soluble factors such as IFNα, IFNγ, latency maintaining factor and nitric oxide (NO) may play a role in initiating latency (136, 152). During this phase, transcription is limited to latency associated transcripts (LATs) (125, 153). In addition, meq has been shown to have a role in maintaining latency as it blocks apoptosis of latently infected CD4+ T cells and transactivate latent gene expression (154).

Beginning 10-14 days post MDV infection, cytolytic infection occurs in the FFE (71). Virus replication in the FFE is fully productive and enveloped cell free virus particles are released (72). The replication occurs regardless of the host resistance and virulence of the MDV strain. MDV most likely transferred to the FFE via infected lymphocytes within lymphoid aggregates that can be seen in perifollicular dermis 7 days post infection (155). The lymphoid aggregates can develop into skin tumor or undergo necrosis. As previously mentioned, FFE infected with fully infectious MDV particles is the main method of transmission to the environment and other birds.

Moreover, susceptible unvaccinated chickens lacking MAb will suffer transient paralysis during latency period (5). Additionally, late-MDV-IS might occur during this period.

1.4.3 Late cytolytic phase

In susceptible birds or resistant birds infected with vv and vv+ MDV, a second wave of cytolytic infection may occur 2-3 weeks after primary infection. The lymphoid organs and most epithelial tissues are often affected during this phase. The bursa and thymus can be atrophied after necrosis of lymphocytes (131). Secondary cytolytic infection does not always occur and depends on host genetic resistance and MDV virulence.

1.4.4 Lymphoma phase

Lymphoma is a one of the consequence of MDV interaction with the host. Mortality due to lymphoma may occur any time after 3 weeks. Although uncommon, in some cases regression of lesions has been reported and depends on the host resistance and age of infection (156-158).

MD tumors consist of mixed cell types including tumor, inflammatory and immunological committed and non-committed cells (159). Majority of the cells in MD lymphomas are T cells although both T and B cells are present (160, 161). Transformed T cells are mainly CD4+ cells expressing TCRαβ1 or TCRαβ2 and MHCII (162) although other subsets such as $CD8^{+}CD4^{-}$, $CD3^{-}CD8^{-}$ and $CD3^{+}CD4^{-}CD8^{-}$ can also be transformed (162, 163). Burgess and Davison (164) showed that MD lymphomas consisting of low and high expressing CD30 cells and $CD30^{hi}$ showed the characteristic of neoplastically transformed cells. Expression of CD30 in B cells has also been reported (22). In addition, MD tumor cells may also express additional antigens such as Marek's Tumor Associated Surface Antigen (MATSA) (165) and fetal antigen (166).

MicroRNA (miRNA) has also been linked with MD tumorigenesis (127, 167-169). MDV-1 miRNAs, mdv1-miR-M4, mdv1-miR-M8 and mdv1-miR-M12 was shown to be highly expressed in tumor cells compared to non-tumorigenic sites in chicken infected with RB1B (167). Morgan, *et al.* (127) reveals that mdv1-miR-M4-5p is the most highly expressed miRNA in MD tumors, representing 70% of miRNA sequencing reads. Moreover, the study also shown that mdv-1-miR-M4 as well as mdv1-miR-M2 were expressed at higher level in lymphomas induced by more virulent virus.

1.5 Factors influencing pathogenesis

1.5.1 Virulence of MDV strain

As previously mentioned, MDV can be classified into pathotypes based on their virulence level (4). The gold standard method for the classification is ADOL pathotyping assay. Higher virulence strain are able to break vaccine immunity conferred by various vaccines which is the basis for current pathotyping assay (4).

Several studies have been conducted comparing different strains on the pathogenesis of MD. Gimeno et al demonstrated that vv and vv+MDV were more neurovirulent than vMDV (5, 45). Highly virulent MDVs was also shown to have faster replication rate and yield higher MDV DNA load in blood than lower virulent strains (12, 46, 149). Moreover, Calnek et al demonstrated that viruses of greater virulence caused more severe atrophy of the Bursa of Fabricius and thymus than less virulent MDVs (12).

1.5.2 Host genotype

Genetic resistance plays an important role prior to introduction of MD vaccine. Two types of genetic resistance have been described: MHC-controlled and non-MHC-controlled

resistance (36). The first evidence for genetic controlled resistance was made by Hutt and Cole (170). Later, Hansen, *et al.* (34) observed association between resistance to MD with B blood group locus. As B blood group locus was a marker for the chicken genes of MHC, genes of the MHC were identified as being associated with resistance (171, 172). Many studies have confirmed that B locus have great impact on the susceptibility towards MDV infection (35, 36, 47-49). Although, results obtained from different studies vary, B^{21} haplotype was shown to confer strong resistance in almost all reports. The $B^{1,}$ $B^{4/}B^{13}$, B^{5}, B^{12}, B^{15} and B^{19} are associated with susceptibility. In most studies, B^{2} are associated with resistance as good as B^{21}, although some reported that it is as low end of susceptibility (173).

Interestingly, chicken of line 6 and line 7 developed at ADOL both have B^{2} haplotype but vary greatly in the MD susceptibility(174, 175). This is a clear evidence that other genes also have strong influence on MD resistance. Studies on these two genetic line revealed that susceptible line had higher viral replication rate from very early stage until death (143, 176).

Although genetic selection for MD resistance became less important after introduction of MD vaccines, it has been found that vaccinated resistant chickens are more protected from MD challenge than their vaccinated susceptible counterparts (52, 53, 177). In addition, previous studies by Bacon and Witter (178, 179) demonstrated that chickens with different B genotypes differ in the development of protective immunity against MD vaccination. Therefore, in addition to vaccination, genetic resistance plays an important role in MD control.

1.5.3 Maternal antibodies (passive immunity)

MAb against MDV protect chicks against several manifestation of MDV infection. Several early studies has shown that MAb are able to reduce early replication of MDV in lymphoid organs, lymphodegenerative syndromes, transient paralysis, and arteriosclerosis (15, 37-39). However, MAb do not prevent either MDV infection or transmission or the development of tumors. Differences in pathogenesis in chickens with and without MAb is presented in Table 1.3.

1.5.4 Vaccinal immunity (active immunity)

MD has been successfully controlled by vaccination since it was first introduced (2, 3, 25, 79). Major vaccines currently being used are HVT (3), HVT+SB1 (26) and CVI988 (Rispens) (25). Vaccine immunity induced by all these vaccines protect against early cytolytic infection and the subsequent lymphoid organ atrophy (147, 180, 181). Additionally, vaccination also prevents the development of transient paralysis (5), lymphodegenerative syndromes (13, 27), panophtalmitis (8), and arteriosclerosis (28). Differences in pathogenesis in chickens with and without vaccination is presented in Table 1.3.

The mechanisms of vaccine induced immunity are only partially elucidated. Payne, *et al.* (32) proposed the concept of “two step” immunity which consist of antiviral and antitumor immune responses. This theory was mainly based on the ability of MATSA to protect against the development of tumors (164, 165, 182-184). However, later studies confirmed that MATSA was not an antigen associated with MD tumors but with activation of T cells, and the theory of anti-tumor immunity lost strength (183). The problem with two step hypothesis is the failure to identify specific tumor antigen. The identification of MATSA by

Witter, *et al.* (165) was thought to represent tumor specific antigen but McColl, *et al.* (185) reported the presence of MATSA on activated T cells. Schat (186) proposed a "one step" protection consisting of only antiviral immune responses. The antiviral immunity against development of lymphoma was confirmed by the efficacy of inactivated viral antigen (187) and recombinant fowl pox virus expressing MDV gB (188) in preventing MD induced tumor. The protection provided by MDV vaccines are demonstrated through cell mediated immunity (189).

Table 1.3: Pathogenesis of Marek's disease in different host condition

DPI	Location	Pathological events	Susceptible without MAb	Susceptible with MAb	Genetically resistant	Vaccinated chickens
0-1	Lung	None	Yes	Yes	Yes	Yes
3-6	Lymphoid organs	Lymphodegenerative syndrome Lymphoid organ trophy	Yes	No	No	No
>7	Blood (viremia)	None	Yes	Yes	Yes	Yes
	Brain	Vasculitis, edema	Yes	No or delayed	No or delayed	No or delayed
	Nerves	Neuritis	Yes	Yes	Yes	Yes
	Eye	Panopthalmitis	Yes	No or delayed	No or reduced	No or reduced
	FFE	Necrosis Full productive infection	Yes	Yes	Yes	Yes
	Other tissue	Necrosis, inflammation	Yes	Yes	Yes	Yes
>21	Various tissues	Neoplasia	Yes	Yes	No or reduced	No or reduced
>15	Blood vessels	Arteriosclerosis	Yes	No	No	No

Adapted from **Gimeno and Pandiri (190)**

1.5.5 Age at infection

Chickens at all age are equally susceptible to MDV infection and are fully susceptible to early cytolytic infection (191-193). However, age plays a role in MD pathogenesis after latency develops. Buscaglia, *et al.* (148) showed that cytolytic infection in older chickens resolved more promptly and the viral load appears to be lower than day-old chicks. Sharma, *et al.* (158) concluded that mechanism for age related resistance was lesion regression, and immunological basis was confirmed in a study using neonatal thymectomised birds. Additionally, Witter and Gimeno (194) demonstrated that adult chickens lacking maternal antibodies and never exposed to MDV until older age develop transient paralysis and B-type lesions in the nerves but no tumors.

1.5.6 Host sex

Various studies reported that females died earlier and experienced higher mortality than males following MDV challenge (32, 33) although opposite results have been also reported (195). The differences are not due hormonal influence and the effect are more pronounced in genetically susceptible chickens and infection with MDV of higher virulence.

1.5.7 Environment and immunocompetence

Various environmental factors such as transport, feed restriction, weather, dehydration and management problems could result in higher incidence of MD (196-198). Stress could result in increase in corticosterone levels, Powell and Davison (199) demonstrated an increase in incidence of MD in chickens experimentally immunosuppressed with corticosteroids. Furthermore, prolonged and more severe early cytolytic infection was observed in chickens experimentally immunosuppressed (148).

Additionally, co-infection with other diseases could exacerbate the effect of the disease due to immunosuppressive effect of MDV (200-202). However, if the co-infective agents are also immunosuppressive, both diseases may be aggravated. This has been observed in concurrent infection with reticuloendothelial virus (REV) (40), chicken infectious anemia virus (CIAV) (41, 43) and infectious bursal disease virus (IBDV) (42, 203).

1.6 Clinical signs and pathobiology

MDV is able to induce various syndromes to the host that can be divided into non neoplastic and neoplastic. Non-neoplastic syndromes are acute transient paralysis (5), lymphodegenerative syndromes (6), panophtalmitis (7, 8), and arteriosclerosis (9, 10). Neoplastic syndromes are associated with development of lymphomas in various tissue. Additionally, MDV can induce immunosuppression that might be have both non-neoplastic or/and neoplastic components.

1.6.1 Transient paralysis (TP)

Two types of TP have been described, classical and acute TP. In classical form, infected birds will recover after suffering of sudden paralysis (38). In acute TP, chickens died within 24-48 hours after the onset of paralysis (5) and it is greatly associated with the pathotype of MDV (50). TP occurs 8-12 days after infection with MDV and either resolve (transient or classical) or results in the death of the chicken (acute) within one or two days(5, 50, 204, 205). TP is characterized by the sudden onset of flaccid paralysis that begins in the neck and later become generalized (38, 206). Acute TP occurs in chickens infected with vv and vv+ MDV (5). The clinical signs of TP are due to the development of vasculitis and vasogenic

edema in the brain (207). Perivascular cuffing, lymphomatosis and gliosis can be also consistently observed in the brain, although such lesions are not always associated with the development of clinical signs (50, 205).

Two syndromes were identified in addition to classical and acute TP (50). These were "persistent neurological disease" (PND) associated with lymphoproliferation and "late paralysis" which is similar to TP but appear 20 days after MDV infection. Gimeno, *et al.* (17) studied MDV replication, cellular infiltration and MHC antigen expression in the brain following v and vv+ MDV. Viral replication of vv+ MDV in the brain was reported to be higher than vMDV. vv+ MDV but not vMDV, induced MHC-I downregulation in the brain, especially at 19-26 dpi. Inflammatory lesions composed of macrophages, $CD4^+$ T cells, $CD8^+$ T cells, started at 6-10 dpi and were transient. In addition, Gimeno, *et al.* (204) demonstrated that there was a differential attenuation of TP and PND induction by MDV, which they proposed to be a useful tool in the study of pathogenesis.

TP only occurs in unvaccinated maternal antibody negative chickens. Nowadays, since vaccines are widely used, the occurrence of this syndrome is not a common phenomenon. However, in countries where vaccination of broilers are not a routine practice, TP might appear in broiler chickens at 30-40 days of age (208).

1.6.2 Lymphodegenerative syndromes

MDV replication in the lymphoid organs (bursa and thymus) resulted in acute cytolytic changes followed by atrophy in these organs (32). No clinical signs are normally associated with this syndrome. However, highly virulent MDV, when inoculated to 1-day-old chickens lacking MAbs, causes severe acute cytolytic infection in the lymphoid organs that results in

death. This syndrome is termed “early mortality syndrome” (6). Gross lesions can be observed as early as 6-8 dpi.

During this phase, abundant viral antigen can be detected in bursa and thymus, especially in the medulla region. Following acute cytolytic phase, infected cells disappear and repopulation of lymphocytes occur. Lymphodegenerative syndromes occur in unvaccinated maternal antibody negative chickens. Since, commercial chickens have MAb and are vaccinated against MD, the relevance of this syndrome is minimal.

1.6.3 Panopthalmitis and ocular disease

Ocular lesions has been associated with infection by vv and vv+ MDV (7, 209). Smith, *et al.* (210) reported transformed cells as early as 11 dpi in the optic and ciliary nerves as well as uvea. However, study by Pandiri, *et al.* (8) classified eye lesions into early and late lesions. Early lesions happen around 6-11 dpi and late around 25-56 dpi. Early lesions include endothelial hyperthrophy, vasculitis and infiltration of lymphocytes (mainly $CD8^{+}$), plasma cells, macrophages, and heterophils involving iris, ciliary body, and choroid layer. Late lesions consisted of severe lymphohistiocytic uveitis, keratitis, pectenitis, vitreitis, retinitis, and segmental to diffuse retinal necrosis. The study also demonstrated that vaccination with HVT protected against early but not late lesion upon challenge with vv+ 648A MDV. Vaccination with CVI988 however protected against both lesions.

1.6.4 Arteriosclerosis

Arterial lesion induced by MDV include proliferative changes in aortic, coronary, celiac, gastric and mesenteric arteries (9, 10, 28, 211). Infiltration of lymphocytes, macrophages and cholesterol deposits in tunica intima and tunica media are among the

common findings. Arteriosclerosis only occurs in unvaccinated MAb negative chickens and the relevance in poultry industry is insignificant (212). Additionally, these lesions in the blood vessels takes a long time to develop and will not occur in the life span of commercial poultry.

1.6.5 Lymphoma

Development of lymphoma is the most relevant MDV-induced syndrome to the poultry industry as it is the cause of condemnation associated with MDV infection. Lymphoma can occur in any organ including ovary, liver, spleen, proventriculus, lungs, adrenal gland, kidney, intestines, skeletal muscle, skin and heart (213). The organ distribution is influenced by host genetics and virus strain. Visceral tumors can appear as diffuse enlargement of the organs and/or as one or more white to grey nodular lesions.

Skin lesions involved feather follicles which can be seen as white colored nodules especially in dressed carcasses (214). Nodular lesions may be seen as scattered lesion or they may coalesce together and form gross nodules. Non-feathered skin can also be affected such as comb, wattles and shanks. Swelling of the wattle or comb may indicate lymphoma growth in underlying tissue. Lesions characterized by cutaneous erythema and reddish discoloration of the skin and shanks commonly known as “Alabama Red Leg” can also be observed (215).

Enlargement of peripheral nerves is an important lesion of MD to differentiate from lymphoid leukosis. Any nerves can be affected. Lesions can be uniform enlargement throughout the nerve or appear as nodules in part of the nerve. Nerve lesions have been classified into three types: A, B, and C (216). Type A are neoplastic lesions characterized by marked infiltration of pleomorphic lymphocytes and the typical composition of MD tumors;

type B lesions are mainly inflammatory and are characterized by infiltration of plasma cells, severe edema, and demyelination of the nerves tissue. Type C lesions are inflammatory but they are much less severe than type B.

1.6.6 MDV-induced Immunosuppression (MDV-IS)

Infection with MDV and development of MD is often associated with immunosuppression (11). MDV-IS can be divided into early immunosuppression associated with viral replication (cytolytic infection) and late immunosuppression that occur when MDV replication is activated and tumors may develop. The early immunosuppression is transient in nature while late immunosuppression is long lasting (permanent) (Figure 1.5).

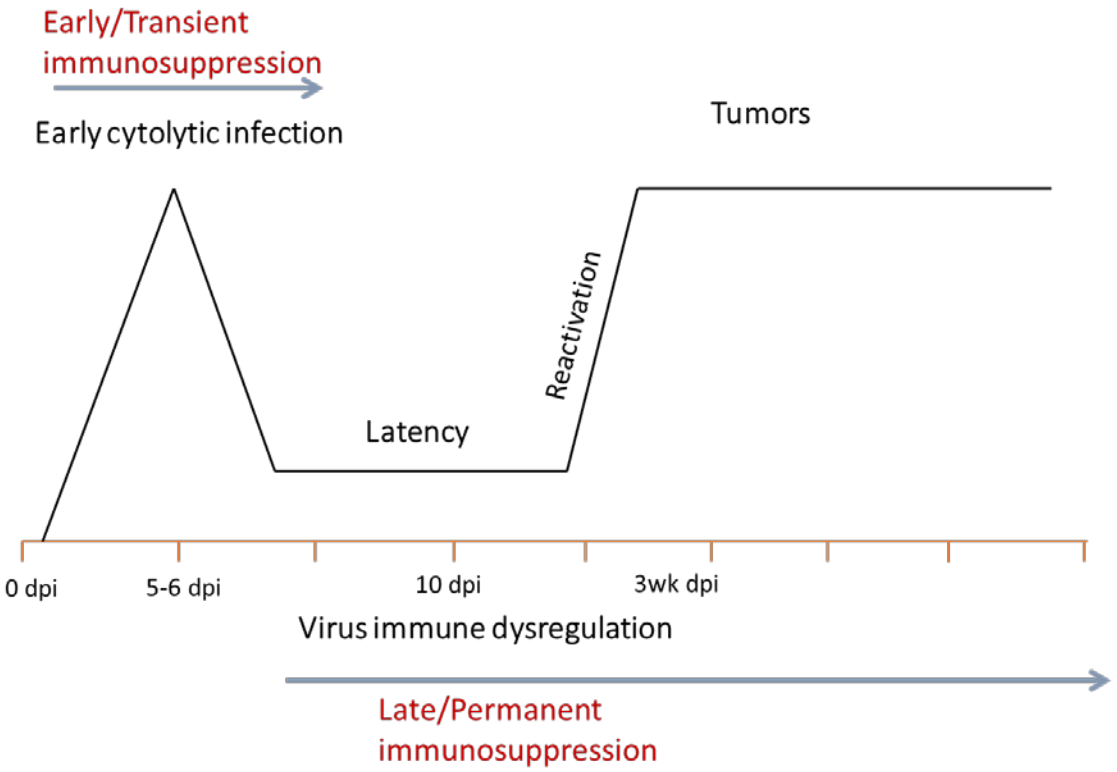

Figure 1.5 Marek's disease induced immunosuppression in the context of MDV pathogenesis. Modified from Gimeno and Pandiri (190).

There are two mechanisms that have been associated with transient MDV-IS: 1) loss of lymphocytes as a consequence of virus replication and 2) presence of suppressor macrophages that could inhibit replication of lymphocytes in vitro. Initial replication of MDV occurs in between 2-4 dpi, which take place in B and some T lymphocyte. MDV cytolytic infection in these cells resulted in their depletion, most likely caused by apoptosis (217, 218). Studies on peripheral blood mononuclear cells shows that CD4+ T cells selectively undergo apoptosis, and CD8+ cells are less susceptible to apoptosis (217, 219). In unvaccinated MAb negative susceptible chickens, replication of vv and vv+ MDV is more and lasts longer than replication of less virulent strains, resulting in more severe lymphoid organ atrophy (12, 149). In contrast to MDV-1, serotype 3 (HVT) and serotype 2 (SB-1) replicate at lower rate in lymphoid organs and causes few or no lesions in the lymphoid organs (220). In addition to viral strains, destruction of lymphocytes also influenced by other factors such as vaccination status, MAb and genetic resistance (Table 1.3). Vaccinated chickens or chickens bearing MAb against MDV do not suffer from early cytolytic infection.

In addition to destruction of lymphoid tissues, it was believed that MDV could induce early-MDV-IS through activation of macrophages. A study by Lee, *et al.* (221) demonstrated that suppressor macrophages were able to inhibit mitogen stimulation of non-infected T cells; however, Schat and Xing (145) suggested that these macrophages are protective rather than immunosuppressive. The inhibition of this response may be the consequence of MDV-induced nitric oxide (NO) production and responsible for reducing the pool of activated T cells thus limiting potential target cells for lytic and latent infection.

Late-MDV-IS is complex and commonly undetected in the field. Several mechanisms may be involved in late-MDV-IS. MDV can regulate the expression of MHC I (16, 17) and MHC II (18). MDV down-regulates cell surface expression of MHC-I protein during active but not latent infection of chicken cells (16). Yu, *et al.* (222) have shown upregulation of MHC-I and β2 microglobulin gene expression during early infection and downregulation at 14 and 28 days post infection. In an in vivo experiment, Gimeno, *et al.* (17) showed that the vv+ MDV strain 648A but not the vMDV strain GA induced severe down-regulation of MHC-I expression on endothelial cells and mononuclear cells infiltrating the brain at 21 days post infection. In addition, infection with vv+MDV was also shown to produce significantly higher level of NO than less virulent strains which could lead to NO-induced apoptosis (19). Recently, microRNA have also been associated with pathogenesis of MDV-IS. Bernberg, *et al.* (20) suggested that overexpression of mdv-miR-M4 in HVT resulted in reduction of commercial vaccine efficiency against *Salmonella Enteritidis*.

Late-MDV-IS occurs concurrent with development of lymphoma, these two manifestation have often been linked. MD tumors express various antigen that can interfere with immune responses such as chicken fetal antigen that interfere with NK killer cells activity and CD30 antigen that may aid in the switch of immune responses toward Th2 response (21, 22). Moreover, addition of MDV lymphoblastoid cells to normal spleen cells was shown to inhibit the proliferation response to mitogen (23). A study by Li, *et al.* (24) demonstrated that deletion of meq reduces the immunosuppressive effect of MDV towards humoral immune response against avian influenza and Newcastle disease. The exact correlation between tumor and late MDV-IS are still unknown.

1.7 Host immune response to MDV infection

Cell-mediated immunity is the main protective immune response to MDV infection. Depletion of either CD4+ or CD8+ T lymphocytes by neonatal thymectomy, and injection of antibodies against CD4 and CD8 molecules in HVT-vaccinated and MDV infected chicken, was shown to prevent tumor development (223). However, virus titers in CD4+ T cells were much higher in CD8-deficient vaccinated chickens than in untreated vaccinated chickens at the early stages of latency, suggesting an essential involvement of CD8+ T cells in anti-virus but not anti-tumor effects (224). Recently, expression analysis in the splenocytes of MVD-infected chickens during early cylytic infection and latency has been published (116). Lysis of infected cells by antigen-specific CTL is the main mechanism of acquired immunity to MDV due to its highly cell-associated nature (225).

The shift from early lytic infection mainly in B cells to a latent phase in the activated T cells, is poorly understood. It is likely regulated by the initial host immune responses to infection. It is speculated that host-virus interaction and subsequent immune responses play a critical role in latency regulation. Studies have shown that impairment of immune responses during the early cytolytic infection delays establishment of latency with an extended lytic phase (226). Cytokines, macrophages, NK cells, cytotoxic T lymphocytes, and antibodies all play important roles in the outcome of MDV infection. Studies show that IFN-γ, IL-6 and IL8 transcription is upregulated in the splenocytes of MD susceptible birds upon infection (139). The transcriptional activities of IL-6 and IL-8 were not detected in MD resistant birds, suggesting that they play a major role in maintaining latent infection in resistant birds and leading to T-cell transformation in susceptible birds. In addition, the expression level of

inducible nitric oxide synthase (iNOS) that catalyzes the production of nitric oxide (NO) is influenced by increased transcriptional activity of IL-8 and IFN-γ, which correlates with the findings that the production of NO by activated macrophages has a direct inhibitory effect on MDV replication (85). A continuous depletion of macrophages over the progression of the disease results in an increased incidence of MD-related complications and tumor development. Chemokines, such as macrophage inflammatory protein 1 β (MIP1β) and K203, are upregulated in resistant chickens which may in turn increase the production of IFN-γ via NK cells (137). NK cells have also been reported to play a major role in controlling early infection through enhanced cell activity and overexpression of NK lysine and granzyme A observed by 3-4 dpi (227).

Although immune responses to MDV infection are primarily cell-mediated, antibody-mediated virus neutralization and antibody-dependent cell cytotoxicity (ADCC) are important components of the humoral immune response. It is important as MDV antigens are expressed on cell surfaces and this components will target infected cells for destruction (37). In previous study, antibodies to MDV glycoprotein B (gB) shown to inhibit virus penetration of host cells, syncytia formation, and cell-to-cell spread (228). However, the effector cells and the antigens of ADCC have not been characterized yet.

1.8 Vaccination

Vaccination is the main control method for Marek's disease in the field. Vaccines have successfully controlled MD since they were first introduced in late 1960s (3, 229). The first vaccine against MD was developed in 1969 via attenuation of MDV-1 strain HPRS-16 (229). It was the first vaccine ever develop against a neoplastic disease. The use of strain

HPRS-16 was soon overtaken by the non-oncogenic strain isolated from turkeys or HVT (3, 62), and from the attenuated MDV-1 strain CVI988, also known as Rispens (25). Currently, there are three main vaccination strategies for MD: attenuated MDV-1, non-pathogenic MDV-2 plus non-pathogenic HVT, or non-pathogenic HVT alone. In addition, recombinant vaccines using HVT as vectors are commonly used worldwide (230-233).

Rispens (MDV-1) provide better protection than vaccines from MDV-2 or MDV-3 strains. Most MD vaccines are cell associated, however HVT vaccines are available as a lyophilized product free of cells or as cell-associated vaccine. In chickens with MAb, cell free HVT provided very low protection due to interference with MAb (234). The route of vaccination also affects protection against MD. Intraperitoneal and intramuscular routes provide better protection than subcutaneous inoculation and intranasal administration provide minimal protection (25, 235). Additionally, in ovo vaccination at 18^{th} day of embryonation provide higher protection against early MDV challenge than vaccination at day of age (236-238).

Other vaccines has been developed using MDV-1 strains, such as US Md11/75C/R2/23 strain (239) and Australia BH16 vaccine (240). The BH16 strain vaccine provides similar level of protection as CVI988 but has not been commercialized. Experimental recombinant vaccines have also been developed for all MDV serotypes (241-243). Recently, a Meq deleted MDV-1 vaccine has been developed and conferred more protection than CVI988 vaccine against vv+ MDV (29-31). This vaccine induces lymphoid organ atrophy in MAb negative chickens and it has not been licensed for commercial use.

There are several vaccine strategies being used to increase the efficacy of the vaccine. In ovo vaccination at 17-19 days of embryonation is currently the most popular method of vaccination, as it is a vastly more time-efficient process compared to manual vaccine injections at hatch (244). Another common strategy consists of using a combination of the two or three strains of various serotypes in bivalent or trivalent vaccine (245). Protective synergism is strong between serotype 2 and serotype 3 vaccine (80). Revaccination consists in administering a second vaccine few days after the first vaccine was administered (246-249). Gimeno, *et al.* (249) demonstrated that in ovo vaccination followed by day old vaccination is the most efficacious revaccination protocol. Adjuvants have also been used to improve the protection provided by MD vaccines. Acemannan, an acetylated mannose polymer was licensed to be used in USA since 1992 but not widely used by the poultry industry (250).

1.9 Diagnosis

MD diagnosis is challenging as there are several tumor diseases in poultry that induce similar lesions. Furthermore infection is not synonymous with disease as most chickens in commercial poultry are infected but will never develop MD. In the differential diagnosis of MD the following diseases should be included: tumors induced by retroviruses (reticuloendotheliosis and avian leukosis virus group), non-neoplastic diseases (hepatitis E, tuberculosis), and diseases that induce neurological clinical signs (i.e. peripheral neuropathy) and/or affect the musculoskeletal system. More details about the differential diagnosis of tumor diseases have been recently published (251).

The diagnosis of MD is a multistep process that requires considering epidemiological, clinical, and pathological data. In some cases, diagnosis can be accomplished at the farm but sometimes it requires to be confirmed by laboratory techniques. The tentative diagnosis can be achieved in the farm by evaluation of flock history, clinical signs, and gross pathology. The confirmative diagnosis can be achieved in the laboratory via evaluation of histopathological lesions, immunohistochemistry and real-time PCR.

Additionally, it is important to note that all currently available diagnostic techniques are used to diagnose MD lymphomas and no diagnostic technique is currently available to detect MDV-IS in the field.

1.9.1 Tentative diagnosis

Age is an important criteria in the diagnosis of MD since MD can affect chickens as early as 3 weeks while disease induced by retroviruses (Reticuloendotheliosis virus (REV) (252) and avian leukosis virus (ALV) (253)) do not occur before 14 weeks of age. However, MD can occur in old chickens as well, and in those cases diagnosis is more complex.

Clinical signs induced by MD can be non-specific but they can also be neurological such as paralysis, ataxia, tremors and torticollis.

Gross lesions induced by MDV can be very helpful in the diagnosis of MD, in particular enlargement of peripheral nerves, skin lesions, and eye lesions as none of them can occur when retroviruses are the etiology of the tumors. However, in the absence of visceral tumors, peripheral nerves lesion can be confused with peripheral neuropathy. Additionally, REV can also induced visceral lymphomas and neuritis which complicates diagnosis for MD (251).

1.9.2 Confirmative diagnosis

Histological evaluation of MD tumors involves assessment of lesion distributions and tumor cell morphology. Lymphoma of visceral organ provides limited value in differentiating between MDV and other retrovirus infection. Lymphoma of visceral organs accompanied with lesions in the peripheral nerves are confirmative of MD. However, it is important to differentiate between types of nerve lesions since type A but not type B is pathognomonic of MD. Type B lesions are inflammatory and are very similar to the lesions of peripheral neuropathy. Additionally, morphology of tumor cells aid the diagnosis of MD. MDV induced lymphomas consist of heterogeneous cell populations of lymphoid cells and macrophages while ALV and REV –induced lymphoma consist of homogenous cell population of lymphoblasts.

Immunohistochemistry is also a valuable approach in the diagnosis of MD. Characterization of tumor phenotypes can differentiate tumors induces by MDV from those induced by ALV and REV. MD tumors are composed of T cells while ALV-induced tumors are composed of B cells. In case of REV-induced tumors, they are usually composed of B cells but they can also be T cells lymphoma (251). Detection of meq oncogene provides a valuable diagnostic tool for diagnosis of MD, but there is no commercially available antibodies against meq oncoprotein at the moment (251, 254).

Quantification of MDV DNA by real-time PCR is the best confirmative diagnosis approach at the moment. Tumor cells can contain up to 100 folds higher levels of MDV DNA than latently infected tissue (254). A technique has been established in our laboratory to diagnose MD via real time PCR and the same assay can be used for early diagnosis of MD

as early as three weeks (254). The diagnosis is based on the Ct ratio. Ct ratio was calculated by dividing the Ct value of housekeeping gene with Ct value of MDV gene amplified. For diagnosis of MDV-1, samples with Ct ratio equal or higher than 1.7 were considered to be compatible with tumors, Ct ratio equal or lower than 1.6 compatible with latency. Cr ratio in between 1.6 to 1.7 is classified as borderline. Diagnosis of MD can be done using samples from tumors, blood and feather pulp (255). Additionally, samples can be kept at -70C or they can be collected and stored in FTA cards until further testing (256).

1.10 Infectious Laryngotracheitis

Infectious laryngotracheitis (ILT) is a contagious respiratory tract infection of chickens that may result in severe production losses. The disease was first described by May and Tittsler in 1925, although it might have existed earlier (257). The disease was identified as avian diphtheria or infectious bronchitis until the term infectious laryngotracheitis was used in 1930 (258). The name infectious laryngotracheitis virus was assigned by the special committee on Poultry Diseases of the American Veterinary Medical Association in 1931. ILTV was the first poultry viral disease for which an effective vaccine was developed.

1.10.1 Etiology

Infectious laryngotracheitis is caused by *Gallid herpesvirus type 1 (*GaHV-1) also known as Infectious laryngotracheitis virus (ILTV) that belongs to the family *Herpesviridae,* subfamily *Alphaherpesvirinae*, and genus *Iltovirus* (259). ILTV is an enveloped virus with linear double stranded DNA genome and has an icosahedral particle with an hexagonal nucleocapsid (80-100nm) similar to other herpesviruses (260). The ILTV genome is approximately 150 kilo base pairs (kbp) in size consisting of 77 predicted ORFs with unique

long (U_L) and unique short (U_S) regions and inverted repeats flanking the U_S region. Five major envelope glycoproteins have been identified, which are responsible for induction of humoral and cell mediated immune responses (261).

1.10.2 Transmission and host

Chickens are the primary host for ILTV although ILT has been described in pheasants, and turkeys. Main mode of transmission of ILTV is horizontal by direct or indirect contact and there is no evidence of vertical transmission (262). Mechanical transmission is the main method to spread the infection and can occur via contaminated equipment, litter, and poultry workers (263). Natural route of entry for ILTV is via upper respiratory and ocular route.

1.10.3 Pathogenesis

ILTV infects mainly upper respiratory tract, lungs, conjunctiva, and air sacs (264, 265). However, ILTV infection can be systemic where the virus spread to liver, cecal tonsils and cloaca. ILTV was shown to infect leukocytes (266) and macrophages in vitro (267) which may explain the possible mechanism for the systemic infection by ILTV. Following infection of the tissue, ILTV begins the lytic phase of replication leading to clinical manifestations. The lytic infection leads to latency of ILTV in trigeminal ganglion (268) coinciding with the stimulation of adaptive immune responses. Any factor that causes immunosuppression such as stress due to introduction of birds or onset of laying reactivates latent ILTV (269). Reactivation of ILTV leads to lytic replication in the epithelium and shedding of the virus via the respiratory route.

1.10.4 Immunity

Humoral and cell mediated immunity (CMI) responses has been described following ILTV infection. Although antibodies are produced against the virus, the humoral immune responses do not influence protection of the host against ILTV infection (270, 271). There is no correlation between the production of local secretory antibodies and regression of clinical signs. In addition, with the use of bursectomized chickens, it has been demonstrated that mucosal antibodies are not essential in preventing viral replication in vaccinated chickens (270). Virus neutralizing antibodies can be detected within 5 to 7 days PI and the highest level of antibody titers is around 21 days PI, however there is no correlation between levels of neutralizing antibodies and resistance to challenge (272). MAb are transmitted from dam to the offspring via the egg; however it does not confer protection to infection or interfere with vaccination (273). On the other hand, CMI is considered the main route of protection against ILTV infection (271, 274). Fahey, *et al.* (275) shows that protection against ILTV infection could be transferred by spleen cells and peripheral blood leukocytes from congenic immune donors, which further verified that CMI responses are the main route of protection.

1.10.5 Clinical signs and pathology

Clinical signs of ILT are generally observed after six to twelve days post infection (276). Clinical signs of severe form are characterized by conjunctivitis, nasal discharge, depression, sneezing, gasping, dyspnea, expectoration of bloody mucous, high morbidity and variable mortality (5 to 70%) (276-279). Cause of death may be the consequence of a decrease on feed intake and mucous plugs in the trachea that leads to suffocation. Gross lesions are characterized by mucoid inflammation, degeneration, and necrosis of the trachea.

Diphtheritic changes may be seen as mucoid casts that fills the entire length of the trachea. Severe hemorrhages into the trachea may result in blood casts, and mucous can be mixed with blood and necrotic tissue (280).

Clinical signs of the milder forms include decreased egg production, weight loss, watery eyes, mild tracheitis, swelling of infraorbital sinuses, persistent conjunctivitis, low morbidity and very low mortality (0.1 to 2%) (281, 282).

The length of infection depends on the severity of lesions, most chickens recover in 10 to 14 days (280). In mild forms of the disease, gross lesions may be seen in the conjunctiva and throughout the respiratory tract, although lesions are more commonly observed in the larynx and trachea, as a mild inflammation or excess of mucous to a severe hemorrhagic tracheitis. In very mild cases, gross lesions may consist only of edema and congestion of the conjunctiva, the infraorbital sinus, and mucoid tracheitis.

Microscopic lesions of ILTV infection depends on the stage of infection. As early as three days post infection intranuclear inclusion bodies are found in epithelial cells and are present only at the beginning of the infection (265, 283, 284). As the infection progresses, epithelial cells in the respiratory tract enlarge, the trachea loses cilia and becomes edematous. The accumulation of lymphocytes, histocytes, and other multinucleated cells form syncytia; plasma cells migrate into the mucus and sub-mucosa after 2 or 3 days post-infection. Late microscopic changes in the trachea can be seen as cell destruction and desquamation of the mucosal surface resulting in the loss of the epithelia covering and leaving a thin layer of basal cells.

1.10.6 Vaccination

Currently, there are two main types of modified-live vaccines commercially available, ILTV attenuated by sequential passages in chicken embryos chicken embryo origin (CEO), or by sequential passages in tissue culture tissue culture origin (TCO). CEO vaccines are live viruses that have been attenuated by repeated passage in embryonated chicken eggs. These vaccines, ideally, do not produce disease, but do cause infection in the trachea, and elicit an immune response (285). Onset of immunity can occur a few days after vaccination, with duration of immunity lasting fifteen to twenty weeks after vaccination, although it can last up to a year (286). TCO vaccines are produced in cell culture to attenuate the virus. TCO vaccines do have a decreased duration of immunity when compared to CEO vaccines (286).

CEO and TCO vaccines are administered via drinking water and coarse spray (287, 288). They can also be administered via infraorbital sinus inoculation (272). Administration of CEO and TCO vaccines practiced mainly in layer or breeder farms. Vaccination is not recommended for broiler type chickens unless other birds in the flock are vaccinated or there is an outbreak (287). ILTV vaccination is expensive to implement for broilers and vaccination can cause reduced performance. In broiler production systems, biosecurity is seen as the main form of prevention for ILT outbreaks. For layer or breeder production systems, CEO and TCO vaccines are administered at 6 to 8 weeks of age, and then again at 12 to 15 weeks. Highest protection occurs from 15-20 weeks, and immunity may vary over the year (286).

Recombinant DNA technology have been used for ILTV and may hold promise for future control and eradication of ILTV. In an approach, ILTV envelope proteins are

expressed in HVT or fowl pox virus as vector. The recombinant virus are shown to protect against very virulent ILTV (289-291).

Even though vaccination is widely used in USA, outbreaks in flocks have been reported in recent years as an emerging problem. There have been an increased number of outbreaks in the Southeastern USA in broiler chickens and vaccination has become generalized. It is hypothesized that the vaccine failure are related to compromised immune system of the host related to immunosuppressive diseases such as MDV.

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CHAPTER 2

Early infection with Marek's disease virus can jeopardize protection conferred by laryngotracheitis vaccines: a method to study MDV-induced immunosuppression[1]

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2.1 Abstract

Marek's disease virus (MDV) is a herpesvirus that induces lymphomas and immunosuppression in chickens. MDV-induced immunosuppression (MDV-IS) is divided into two phases: early-MDV-IS occurring mainly in chickens lacking maternal antibodies (MAb) against MDV and associated with lymphoid organ atrophy; and late-MDV-IS occurring once MDV enters latency and during tumor development. Our objectives were to document the impact of late-MDV-IS on commercial poultry (meat type chickens bearing MAb against MDV and that were vaccinated or unvaccinated against MD) and to optimize a model to study late-MDV-IS under laboratory conditions. The impact of late-MDV-IS was evaluated by assessing the effect of early infection (day of age) with a very virulent plus MDV (vv+MDV) on the efficacy of chicken-embryo-origin (CEO) infectious laryngotracheitis (ILT) virus vaccine against ILT challenge. The CEO ILT vaccine was administered in water at 15 days of age and ILTV challenge was done intratracheally at 30 days of age. Development of ILT was monitored by daily evaluation of clinical signs, development of gross and histological lesions in trachea, and quantification of ILT virus (ILTV) transcripts in trachea. Infection with vv+MDV strain 648A resulted in total abrogation of protection conferred by the CEO vaccine against ILTV challenge even in chickens vaccinated at day of age with either HVT, HVT+SB-1, or CVI988. Chickens exposed to vv+MDV prior to vaccination with CEO ILTV vaccine had similar ($p<0.05$) clinical scores, gross lesions, histopathologic lesion scores, and load of ILTV transcripts in trachea after ILTV challenge, as chickens that were not vaccinated with CEO ILTV vaccine.

2.2 Introduction

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by the oncogenic herpesvirus, Marek's disease virus (MDV), also known as *Gallid herpesvirus* 2 (GaHV-2). MD is a major threat to the poultry industry because in the absence of control methods it leads to severe economic losses. MD and associated economic losses have been successfully controlled by vaccination since 1969 (1, 2). However, MD still has a significant economic impact due to sporadic outbreaks that still occur in vaccinated flocks and because of the cost associated with vaccines and administration of vaccines. Various vaccines have been developed to control MD, the most effective of which is CVI988 (3, 4); however, the efficacy of MD vaccines tend to decrease with time as MDV evolves towards more virulence (5). Some emerging MDV strains are able to break the immunity conferred by currently available vaccines. Based on the strain's ability to break vaccine immunity, isolates have been classified into different pathotypes (virulent (vMDV), very virulent (vvMDV), and very virulent plus (vv+MDV) (5). Increase in virulence is not only limited to the breaks in vaccine immunity but also to an increase in neurovirulence (6). In addition, the vv+ strains cause more severe damage to lymphoid organs which is probably leads to increased immunosuppression than the less virulent pathotypes (7).

Marek's disease virus-induced immunosuppression (MDV-IS) pathogenesis is highly complex and poorly understood. MDV-IS is often divided into two phases: an early immunosuppression phase associated with early cytolytic infection of the lymphoid organs (early MDV-IS); and a late immunosuppression phase associated with the establishment of

latency and development of tumors (late MDV-IS) (7, 8). Early-MDV-IS is due to the destruction of the lymphoid organs during the 5-6 days following infection, which leads to a varying degree of atrophy of the bursa of Fabricius and thymus. Severity of lymphoid organ atrophy depends on the pathotype of MDV and can last for long periods of time when MAb- chickens are infected with highly pathogenic MDV (7, 9). Early-MDV-IS does not occur in chickens bearing MAb or in chickens that are vaccinated against MD (10-12). Islam et al (2002), however reported lymphoid organ atrophy in MAb+ broiler chickens challenged with Australian vvMDV strain MPF 57 (13).

The late MDV-IS (late-MDV-IS) is believed to be permanent or at least long lasting (14). Mechanisms are poorly elucidated and seem to go beyond destruction of lymphoid organs, although reactivation from latency could also lead to lysis of lymphoid cells (15). *In vitro,* MDV down regulated cell surface expression of MHCI protein during cytolytic infection of chicken fibroblast cell line OU-2 and during reactivation from MSB-1 and UA04 cell lines (16). In an *in vivo* experiment, Gimeno et al have shown that vv+ MDV strain 648A but not vMDV strain GA induced severe down-regulation of MHC class I expression on endothelial cells and mononuclear cells infiltrating the brain starting at 21 days after infection (17). Morimura et al (18) demonstrated that MDV infection could down regulate CD8 molecule expression in peripheral T cells and induce apoptosis of $CD4^+$ T cells, which could contribute to MDV-IS. Tumor cells were also suggested to be responsible for the immunosuppression: Quere (19) has shown that MDV-transformed T-lymphoblastoid cell lines suppress the lymphoproliferative response to T mitogens (concavanalin A) of chicken splenic lymphocytes *in vitro;* Islam et al (13) and Cui (20) also demonstrated that MDV-

induced tumors can negatively affect humoral immune responses; finally tumors induced by MDV have also been known to induce immunosuppression through expression of antigen such as chicken fetal antigen that interferes with activity of natural killer cells (21). It is likely that some of these mechanisms contribute to some degree to MDV-IS.

Infectious laryngotracheitis (ILT) is a highly contagious respiratory disease of chickens that produces significant economic losses to the poultry industry. Frequency of ILT has increased in recent years in the Southeastern USA. Administration of modified live vaccines (i.e. chicken embryo origin or CEO) protects against the development of the disease but has been related to perpetuation of outbreaks due to viral persistence, virus shedding, spread to unvaccinated birds, and reversion to virulence. Recombinant DNA-derived vaccines using HVT or fowlpox as vectors have been produced and are commercially available but their efficacy seems to be lower than that achieved by modified live vaccines (22). In the USA, the most common vaccination protocols in broiler chickens is CEO ILTV vaccine in the drinking water. Even though CEO has been associated with reversion to virulence (23), it is still considered to be the most protective ILT vaccine that can be mass-administered (22). Several explanations are possible for the increased incidence of ILT in broiler chickens in the USA. Poor biosecurity and uneven vaccination by using CEO ILT vaccine in the drinking water are well recognized (24). Our hypothesis is late-MDV-IS is capable to reduce protection induced by ILT vaccines (CEO ILT), and could be at least partially responsible for the recent outbreaks of ILT.

The impact of late-MDV-IS in commercial poultry is unknown. There are no currently available and accepted methods for detection of late-MDV-IS in the field. There might not be lesions associated with late-MDV-IS since it can occur in the absence of lymphoid organ atrophy. It is demonstrated that tumors can induce MDV-IS, however it is uncertain if late-MDV-IS could occur in the absence of MD tumors or if presence of tumors is always associated with immunosuppression. There are many mechanisms by which MDV can induce immunosuppression but it is unknown which one of them is relevant in commercial chickens bearing MAb and/or vaccinated, and therefore have any practical relevance. The principal impediment to study late-MDV-IS is its complexity and a need of a model that is reproducible under laboratory conditions. The objectives of this study were to demonstrate the impact of late-MDV-IS on the efficacy on ILT vaccination in commercial poultry (meat type chickens bearing MAb against MDV that either were unvaccinated or had been vaccinated against MD at day of age) and to develop a model that would allow us to reproduce late-MDV-IS under laboratory conditions. Once a model is established, further studies will be warranted to evaluate mechanisms associated with the detected immunosuppression and to develop methods of detection and control.

2.3 Materials and Methods

Chickens. Commercial specific pathogen free SPAFAS chickens (Charles River SPAFAS, N Franklin, CT) were used as MDV shedder chickens. Female commercial meat type chickens (grandparents) bearing MAb against MDV were used as experimental chickens. In the past, female chickens have been shown to be more susceptible to MD than males (25).

Furthermore, in a previous experiment using the same genetic line, we have shown that females were more susceptible to MD after infection with vv+MDV strain 648A than males (26). Experimental chickens came from dams that were vaccinated with vaccines of the three serotypes (HVT, SB-1, and CVI988), therefore they were considered to have MAb against the three serotypes.

Viruses and vaccine. MD vaccines strains CVI988 (3), SB-1 (27), and HVT (28) were used. Serotype 1 MDV strain, 648A (vv+) (5) at passage 12 in chicken embryo fibroblast (CEF) was used as the MDV challenge. Chicken embryo origin (CEO) vaccine strain of ILTV was obtained from a commercial manufacturer (Merial Select, Inc., Gainesville, GA, USA). Illinois-N71851 strain of ILTV that has been characterized as a virulent ILTV strain was used for infectious laryngotracheitis (ILT) challenge (29, 30).

Experimental Design. Two experiments were conducted using commercial female meat type chickens as experimental chickens and SPAFAS chickens as shedders. Animal experiments were conducted following the guidance and under approval of North Carolina State University Institutional Animal Care & Use Committee (IACUC). SPAFAS chickens were vaccinated with HVT vaccine *in ovo* via amniotic route at 18 days of embryonation (ED) to avoid the development of transient paralysis and to ensure survival. At hatch, shedder chickens were infected with 500 PFU of vv+MDV 648A strain subcutaneously and housed in isolation for 15 days prior to the beginning of experiment. In each floor pen, 15-day-old shedder chickens were comingled with 40-42 one-day-old meat type experimental chickens (see below) for infection of MDV by contact. Shedders were maintained for 4

weeks, then euthanized and subjected to necropsy. All shedders in both experiments either died of MD or had MD lesions at 4 weeks of age. The in-contact birds consisted of 4 groups subjected to the following treatments: group 1 received no treatment; group 2 was challenged with ILTV; group 3 was vaccinated with CEO and challenged with ILTV; group 4 was infected by contact exposure with MDV, vaccinated with CEO ILT vaccine and challenged with ILTV. In addition, experiment 1 included 3 groups of chickens (groups 5, 6, and 7) that had received 2000 PFU of HVT, HVT+SB-1, or CVI988, respectively, via subcutaneous route at day of age, challenged with vv+MDV 648A by contact, vaccinated with CEO ILTV vaccine, and challenged with ILTV. The number of chickens per treatment group for both experiments 1 and 2 were between 40 and 42 chickens. All in-contact chickens were wing-banded at hatch and placed in environment controlled rooms (BSL-2) for the rest of the experiment.

CEO ILTV vaccine was administered to the experimental chickens (meat type) at 15 days of age via drinking water as per manufacturer's recommendations. ILTV challenge (2,000 PFU) of the meat type chickens was done intratracheally at 30 days of age.

All chickens were monitored for clinical signs for 6 days between challenge with ILTV (30 days of age) and the termination of the experiment (36 days of age). In experiment 1, chickens were not identified with leg bands and individual monitoring of clinical signs was not done. In experiment 2, each chicken was identified with colored leg bands and clinical signs were recorded daily for each individual chicken. At days 4 and 6 (termination) following ILTV challenge, 6 chickens per group were weighted, euthanized, and tissue

samples were taken for real time RT-PCR (trachea) and histopathology (trachea, bursa and thymus). Weights of bursa of Fabricius and thymus were also obtained. Chickens euthanized for sampling at 4 and 6 days post inoculation (dpi) were randomly selected. All chickens were necropsied at termination (36 days of age) or at time of death and examined for gross lesions of ILT and MD.

Clinical Signs. In experiment 1, clinical signs could not be evaluated for individual birds due to lack of color leg bands. Birds in experiment 2 however were individually observed daily for clinical signs of ILT including gasping, coughing, sneezing, conjunctivitis and expectoration. Mortality was also recorded.

Two methods were used for scoring the severity of ILT: intratracheal pathogenicity index (ITPI) and clinical sign score (CS score). Both methods involved evaluating clinical signs daily in each individual chicken for 6 days after the challenge with ILTV. ITPI was calculated as reported by Guy et al (31). Briefly, each chicken was scored daily as being normal (score 0), have respiratory clinical signs (score 1), or have died as a result of disease (score 2). The record of the status of each chicken each day is considered to be a subjective observation. Therefore ITPI was calculated for each group as the sum of scores for all chicken for the 6 days divided by the total number of observations. The CS score was calculated for each chicken as the sum of days a particular chicken showed clinical signs. The CS score was used to calculate ILT index (see data analysis and statistics).

Gross lesions. Upon necropsy, dead birds were evaluated for ILT and MD lesions. ILT gross lesions were scored from 0 to 4 with as follow: 0 = normal; 1 = light mucus; 2 = congestion, thick mucus or bloody mucus; 3= caseous exudate; and 4 = plugs.

For MD lesions, the presence of tumors in visceral organs, typical MD ocular and skin lesions, and/or enlargement of sciatic nerves were recorded.

Relative lymphoid organ weight. To determine bursa and thymus weights relative to total body weight, body weight and lymphoid organ weight was evaluated in 6 chickens per treatment group on the day of termination of the experiment (day 7 post ILTV challenge).

Histology. Trachea, thymus and bursa of Fabricius were collected for histopathology from 6 chickens per treatment group at 4 and 6 days after infection with ILTV. The tissues were fixed in 10% neutral buffered formalin, routinely processed and stained with hematoxylin and eosin. An upper portion of trachea (1cm from larynx) was sampled for histopathology. Microscopic lesions in trachea were scored on a scale of 0-5 (normal to very severe) using previously described criteria (31). Bursal lesions evaluated included lymphoid necrosis with depletion, cyst formation, and follicular atrophy. Thymic lesions evaluated included necrosis, lymphoid cell depletion and decrease cortex to medulla ratio.

Atrophy of lymphoid organs was also evaluated by image analysis. Image J (National Institute of Health, Bethesda, Maryland, USA) software was used to calculate a mean gray scale value for thymus and bursa of Fabricius samples. A digital image of representative fields of each lymphoid organ was taken and analyzed. A diagonal line was drawn across each digital image and the grayscale value of each pixel on the line was measured. The

grayscale values range from 0 = black to 255 = white, which estimate the density of the lymphoid cell population. Loss of lymphocytes would results in a higher mean gray scale value (32-34).

Real time RT-PCR. Tracheal mucosa was scraped and placed into 1ml cold (4°C) sterile PBS containing 100 µg/ml gentamicin and 5 µg/ml amphotericin B. RNA was extracted from tracheal swab washes using the Perfect Pure RNA tissue kit (5-Prime Inc, Gaithersburg, Maryland, USA) following manufacturer's recommendation. Real time reverse transcriptase PCR (real time RT-PCR) was performed to measure the transcription of ILTV gI gene using the following primer sequence: forward (F) 5'-TGTGGGCAGTGGACTATGTT-7' and reverse (R) 5'-TCAGGGTCAGCAAGTATTGG-7'(35). Samples were amplified using two primer sets specific for ILTV gI genes and for housekeeping gene 28S rRNA with the following sequence: forward (F) 5′-GGCGAAGCCAGAGGAAACT-3′ and reverse (R) 5′-GACGACCGATTTGCACGTC-3′ (36). Amplifications were done in an Mx3005 Stratagene (Stratagene, La Jolla, California, USA) thermocycler by amplifying 25µl reactions using Brilliant II SYBR® Green Q-PCR Master Mix (Agilent Technologies, Santa Clara, CA, USA). The profile cycles used were 1 cycle of 50°C for 30 min; 1 cycle of 95°C for 10 min; 50 cycles of 95°C for 10 sec and 56°C for 1 min; and 1 cycle of 95°C for 1 min, 55°C for 30 sec, and 95°C for 30 sec. The melting curves were plotted at the completion of amplification steps by cooling the sample at 2.0^{o}C/s to 60^{o}C and then increasing the temperature to 95^{o}C at 0.1^{o}C/s. Analysis of the data was done as described previously (37). Samples were tested in duplicates and the Ct values were averaged (Ct mean). For each sample, Ct mean values were subtracted from total number of cycles (50 –Ct mean).

Correction for differences in RNA levels between samples was done by using a 28S correction factor as follow:

28S correction factor = 50 - Ct mean value for 28S rRNA-specific product from all samples) / 50 - Ct mean value for 28S rRNA-specific product for each sample.

Since efficiency of the reactions for the 28S and for the evaluated viral gene might differ, a corrected viral gene means was calculated based on the slope of the standard curves for each reaction as follow:

Corrected viral gene means = 28S correction factor X (50 - Ct mean value for viral gene) X viral gene slope/28S gene slope)

Data analysis and statistics. Data were analyzed using statistical program Statistica (Stat Soft, Tulsa, Oklahoma, USA) and SPSS (IBM, New York, USA). Comparison between percentages (% chickens showing clinical signs, % chickens showing ILT gross lesions, % chickens showing MD lesions) was done by the Z-test using Statistica. Comparison of more than two groups with continuous variables (ITPI values, relative lymphoid organ weight, lymphoid atrophy results obtained derived from the image analysis) was conducted by one-way analysis of variance (ANOVA) test using SPSS. The Scheffe test was used as a post hoc analysis. Comparison of categorical variables (CS scores, histopathological results of lesions in trachea) was conducted by Kruskal–Wallis test using SPSS. P values of <0.05 were considered significant.

Three indices (ILT index, protection index and immunosuppression rank) were calculated based on scores for clinical signs and gross lesions in each chicken in experiment 2. Number of days exhibiting clinical signs (CS) was calculated for each chicken (values 0-6). Gross lesions (GL) in trachea at termination were scored based on severity (values 0-4). ILT index was calculated to assess severity of the disease based on CS and GL.

ILT index (ILTI) = CS + GL (values 0-10)

Protection index was calculated using ILTI values from the positive control group (-/ILTV) and the CEO ILTV vaccinated groups (-/CEO/ILTV, and MDV/CEO/ILTV) to measure protection provided by the CEO ILTV vaccine.

Protection index (PI) = $(ILTI_{-/ILTV} - ILTI_{TXT}) / ILTI_{-/ILTV}) \times 100$

$ILTI_{-/ILTV}$ is ILT index of -/-/ILTV control group.

$ILTI_{TXT}$ is ILT index of any treatment group that had been vaccinated with CEO ILTV vaccine.

Immunosuppression rank was calculated using PI values of the -/CEO/ILTV and MDV/CEO/ILTV to evaluate the effect of late-MDV-IS on protection induced by CEO ILTV vaccination.

Immunosuppression rank (IS rank) = $100 - ((PI_{MDV/CEO/ILTV} / PI_{CEO/ILTV}) \times 100)$

2.4 Results

ILT clinical signs. The percentage of chickens that developed ILT clinical signs between days 1 and 6 after ILTV challenge as well as the ITPI and CS scores are presented in Table 2.1. The highest frequency of ILT clinical signs occurred at day 6 post ILTV challenge regardless of the treatment group. All chickens in group -/-/ILTV and 73% of chickens in group 648A/CEO/ILTV showed clinical signs at day 6 post ILTV challenge. The frequency of chickens showing clinical signs at 6 days post ILTV in the CEO ILTV vaccine control group (-/CEO/ILTV) (12%) was significantly lower ($p<0.05$) than in groups -/-/ILTV and 648A/CEO/ILTV.

Group-/-/ILTV had the highest ITPI (0.56) followed by group 648A/CEO/ILTV (ITPI =0.5). Group-/CEO/ILTV had the lowest ITPI (0.04). The same trend was observed for the CS scores which represent the average number of days animals showed clinical signs in each group. Negative control group (-/-/-) scored 0. Group -/-/ILTV had the highest CS score (4.36), followed by group 648A/CEO/ILTV (3.61). Group-/CEO/ILTV had the lowest CS score (0.21).

ILT and MD gross lesions. ILT and MD gross lesions were evaluated in every chicken after death or at the termination of the study (36 days). The frequency of chickens developing gross lesions (either MD, ILT, or both) and the ILT gross lesions scores are shown in Table 2.2. In experiment 1, chickens challenged with 648A developed as higher frequency of ILT lesions as the group -/-/-/LTV (50%), regardless of being vaccinated at day of age with HVT (70%), HVT+SB-1 (63%), CVI988 (63%), or remained unvaccinated (50%). All groups

challenged with 648A had higher frequency of ILT lesions than the control group -/-/CEO/ILTV (13%). The same trend was observed regarding the ILT gross lesions score. MD vaccination reduced the frequency of MD tumors but it did not reduce the negative effect of 648A on the efficacy of CEO against ILTV challenge. In the groups challenged with 648A, the frequency of ILT lesions in chickens without MD tumors was higher in the groups that received MD vaccines (32 to 46%) than in the unvaccinated group (11%). In experiment 2, 100% of the chickens in group -/-/-/ILTV but none of the chickens in group -/-/CEO/ILTV developed ILT lesions. Group challenged with 648A had 41% of the chickens with ILT lesions (19% only ILT and 28% both ILT and MD) and was significantly different than all control groups.

ILT microscopic lesions: Figure 2.1 summarizes microscopic lesions in the trachea of chickens necropsied at 4 and 7 days post ILTV exposure (6 chickens per treatment and time point) in experiments 1 and 2 for groups 1-4. Chickens in group -/-/- presented no microscopic lesions in trachea in any of the experiments or time points. Group -/-/ILTV developed the most severe average lesions (score 5 and 4.9 at day 4 and 4.1 and 5 at day 7 in experiments 1 and 2, respectively). Group 648A/CEO/ILTV had lower, although not significantly (except 7 dpi of experiment 2), average lesions (4.4 and 3.9 at day 4 and 3.7 and 2 at day 7 in experiments 1 and 2, respectively). Group -/CEO/ILTV had significantly lower average lesion scores (2 and 0.3 at day 4 and 2.7 and 1.67 at day 7 in experiments 1 and 2, respectively) than groups -/-/ILTV and 648A/CEO/ILTV.

ILTV gI transcript. Results of the detection of ILTV gI transcripts are shown in Figure 2.2. No ILTV gI transcripts were detected in the tracheal swabs of groups -/-/- and -/CEO/ILTV. In group -/-/ILTV, all 6 sampled chickens had detectable levels of gI transcripts with a relative mRNA expression normalized to 28S of $1.2x10^6$. In group 648A/CEO/ILTV, 4 out of 6 chickens had detectable levels of gI transcripts and the viral load was not statistically significantly different compared to group -/-/ILTV (relative mRNA expression normalized to 28S of $1.0x10^6$).

Lymphoid organ atrophy. Results of lymphoid organ atrophy as determined by lymphoid organ weight to body weight ratios, histopathology, and mean gray values are presented in Table 2.3. No evidence of bursa and thymus atrophy due to MDV challenge was detected by any of the three parameters when compared to control unvaccinated chickens. However, relative thymus weight were statistically ($p<0.05$) lower in group -/-/ILTV (0.16) compared to other treatment groups (-/-/- =0.49; -/CEO/ILTV = 0.48; and 648A/CEO/ILTV =0.40) although no differences were found by histopathology and image analysis.

Indices to evaluate late-MDV-IS in ILT model. ILT index, protection index, and IS rank were calculated in experiment 2 to simplify the evaluation of late-MDV-IS in the ILT model. The formula to calculate those indices is presented in Materials and Methods. Results are presented in Table 2.4. LT index value was highest for group -/-/ILTV (7.64 out of 10), followed by group 648A/CEO/ILTV (5.46 out of 10), and had the lowest values for group -/CEO/ILTV (0.79 out of 10). Protection index indicates the ability of CEO to protect against ILTV challenge. Group -/CEO/ILTV had PI values of 89.65. Previous infection with MDV

(648A/CEO/LVT) decreased the ability of CEO to protect to 28.53%. The IS rank for the 648A strain used in this study was calculated at 68.2.

2.5 Discussion

We developed a model (late-MDV-IS ILT model) to demonstrate and reproduce, under laboratory conditions, the impact of late-MDV-IS on commercial chickens bearing MAb against MDV and that were unvaccinated against MD or vaccinated at day of age with HVT, HVT+SB-1, or CVI988. Our result shows that early infection with vv+MDV strain 648A can abrogate protection conferred by a CEO ILT vaccine against a virulent ILTV challenge. This model used commercial chickens bearing MAb against MDV, the natural route of infection for MDV, and the route and schedule of vaccination for ILT in meat type chickens (CEO ILTV vaccine in drinking water at 14 days of age). Therefore, it is possible that similar situations occur under field conditions and late-MDV-IS might have contributed to the ILT outbreaks reported in recent years (38-40). Furthermore, our results demonstrated that late-MDV-IS occurs in the absence of lymphoid organ atrophy and can occur in chickens lacking gross tumors, which complicates the diagnosis of MDV-IS. MDV is a known potent immunosuppressive agent (41). Nonetheless, MDV-IS has been poorly studied mainly due to the complexity of mechanisms involved. Results of our study provide a standardized model to study late-MDV-IS. Our model is reproducible and its use should aid in better understanding of the mechanisms involved in late-MDV-IS as well as in developing methods of detection and control.

MDV induces two phase of immunosuppression. The first phase of immunosuppression (early-MDV-IS) is a consequence of the replication of MDV in the lymphoid organs resulting in lysis of lymphocytes. The duration of this phase is variable and seems to last longer when highly virulent MDVs are involved (7, 9, 42). The second phase of immunosuppression (late-MDV-IS) occurs after the virus goes into latency and it is thought to be long-lasting (14). Early-MDV-IS can be controlled by the combination of MAb against MDV and vaccination thus transient MDV-IS is not considered to have much relevance under commercial conditions (10, 12, 43). Late-MDV-IS is more complex and poorly understood. In the present study, we have demonstrated that it is not prevented by MAb as it occurred in commercial chickens derived from hens vaccinated against the three serotypes of MDV. Furthermore, in this study vaccination with HVT, HVT+SB-1, or CVI988 at day of age could not protect against the negative effect of 648A on the efficacy of CEO ILTV vaccine. In the present study, evaluation of MDV-IS in vaccinated chickens was very limited (only one replicate using 1 day old vaccination). Further studies to evaluate if other more protective MD vaccination protocols (i.e. in ovo vaccination, higher dose of vaccine, double vaccination) can control MDV-IS are warranted. Also, the ability of MD vaccines to induce immunosuppression on their own need to be evaluated.

The late-MDV-IS ILT model indirectly evaluates late-MDV-IS by assessing the effect of MDV infection on the efficacy of CEO ILTV vaccine. ILT is a respiratory disease induced by an alpha-herpesvirus (*Gallid herpesvirus* 1) (44). ILTV infection is localized in the upper respiratory tract and produces ready detectable respiratory clinical signs which makes observation and monitoring of the disease relatively simple. Immunity against ILTV

is based on cellular immune responses (45), therefore the ILT model focuses on the effect of MDV on cell mediated immunity. In this study, we have optimized the model by improving the techniques used to evaluate and monitor different aspects of ILT. Results obtained from observations of respiratory clinical signs, tracheal gross lesions, microscopic lesions in trachea, and the presence of gI transcripts in tracheal washes were consistent and strongly correlated. Therefore it was concluded that clinical signs and gross lesion are sufficient parameters to use in evaluating the ILT outcome in the late-MDV-IS ILT model. Both clinical signs and gross lesions can be evaluated in every animal and do not require expensive laboratory procedures. Evaluation of clinical signs has to be done for each chicken although it is critical that chickens are individually identified for clinical observations. The use of colored leg bands facilitates visual identification of each chicken. Data obtained from clinical signs and gross lesions were combined to develop the ILT index that provides a measure of the level of ILT in a given chicken. Furthermore, a protection index was calculated to evaluate the level of protection that CEO ILTV vaccine conferred in a particular group. Finally, an IS-rank was calculated to evaluate the effect of MDV on the efficacy of CEO ILTV vaccination against ILT.

In this study, vv+MDV strain 648A was used. Previous work has demonstrated that pathotype might have an effect on early-MDV-IS (7). Furthermore, differences were found between vv+ strain 648A and vMDV GA in the ability to down-regulate MHC-I in endothelial cells and mononuclear cell infiltrates in the brain (17), and in the ability to induce NO (46). Further studies are warranted to evaluate the ability of other MDV pathotypes to

induce MDV-IS and to elucidate the possible role of increased immunosuppressive ability in the evolution of MDV.

Understanding the mechanism by which vv+MDV induces late-MDV-IS in the ILT model is critical to find methods of diagnosis and control. Immunity against ILTV is based on cellular immune responses (45), therefore the ILT model focuses on the effect of MDV on cell mediated immunity. MDV might affect cell mediated immunity through different mechanisms. MDV can down-regulate MHC-I (16, 17) and dysregulate cytokines required for cytotoxic T cell function (36). Transformed lymphocytes might have a role in MDV-IS (21). At this point, it is unknown if the immunosuppression evaluated in the late-MDV-IS ILT model is related to the development of tumors. In the present study some chickens were immunosuppressed although no gross tumors were observed. Vaccination against MD at day of age reduced the development of tumors but it did not reduce the development of MDV-IS. Our results are suggestive that late-MDV-IS might not be associated with tumors but further studies need to be done to confirm if this is the case. Our results contrast with the work of Islam et al (13) reported protection against MDV-IS by HVT in broiler chickens. The fact that chickens developed lymphoid organ atrophy in the work of Islam et al (13) might indicate that they suffered from early-MDV-IS and HVT protected against it. In our study we could not detect lymphoid organ atrophy even though the challenge virus that we used was a vv+MDV. It is possible that differences could be due to variation in genetics or in the level of MAb (we used grandparent meat type chickens and Islam et al. used commercial broiler chickens). Differences in the challenge (ours was done by contact and Islam et al. was done by inoculation) might have also contributed for the different results.

This study demonstrates that late-MDV-IS can occur in commercial chickens bearing MAb against MDV; it is not related to lymphoid organ atrophy; and can occur in the absence of gross MD tumors. Because of the economic repercussions of late-MDV-IS for the poultry industry, further studies are needed to find proper methods of diagnosis and control. This study has provided the first step to study late-MDV-IS by standardizing a model (late-MDV-IS ILT model) that allows reproducing late-MDV-IS under laboratory conditions,

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2.7 Figures and tables

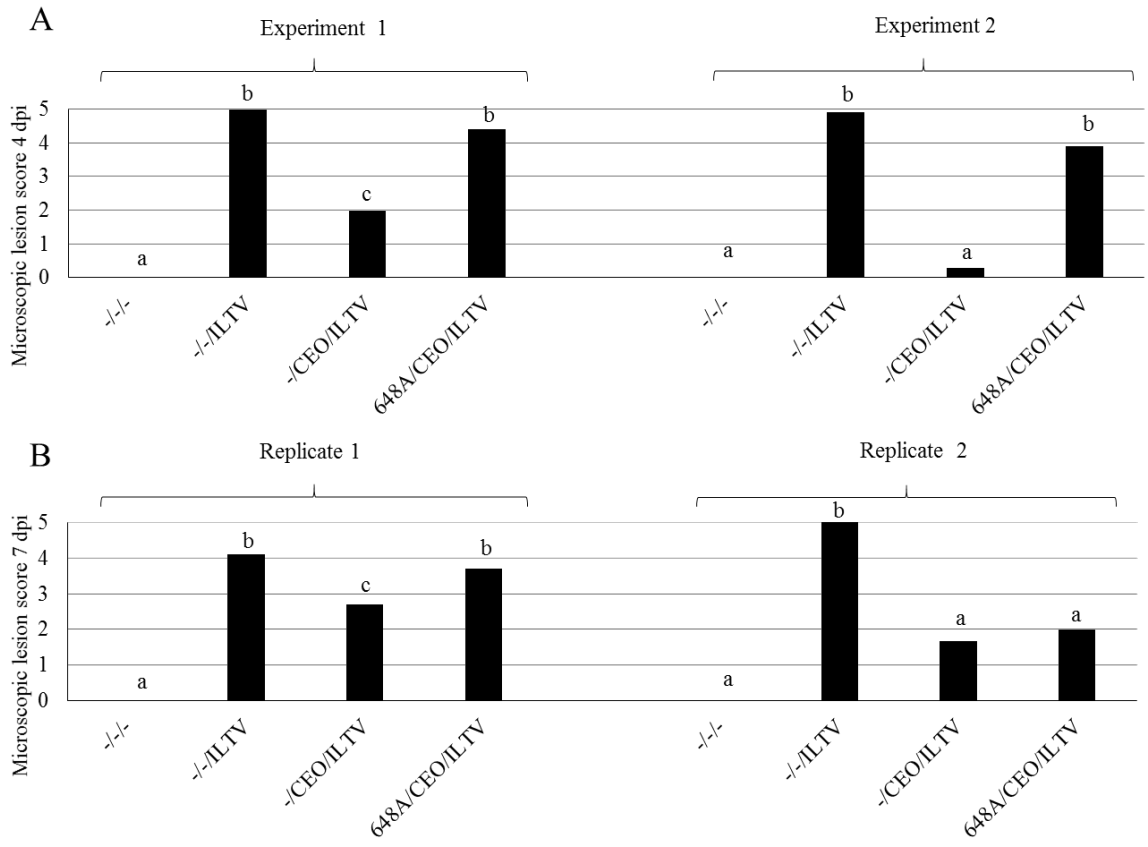

Figure 2.1: Tracheal microscopic lesions at (A) day 4 and (B) day 7 post ILTV challenge (6 chickens per treatment group). Results of two experiments are presented. The average of the microscopic lesions score per treatment group with a range from normal (0) to most severe (5) is shown. Different letters over the bars indicate statistically significant ($p<0.05$) differences. Group -/-/- received no treatment (negative controls); Group -/-/ILTV received only ILTV challenge; Group -/CEO/ILTV received CEO vaccination and ILTV challenge and; Group 648A/CEO/ILTV infected with MDV, vaccinated with CEO and then challenged with ILTV.

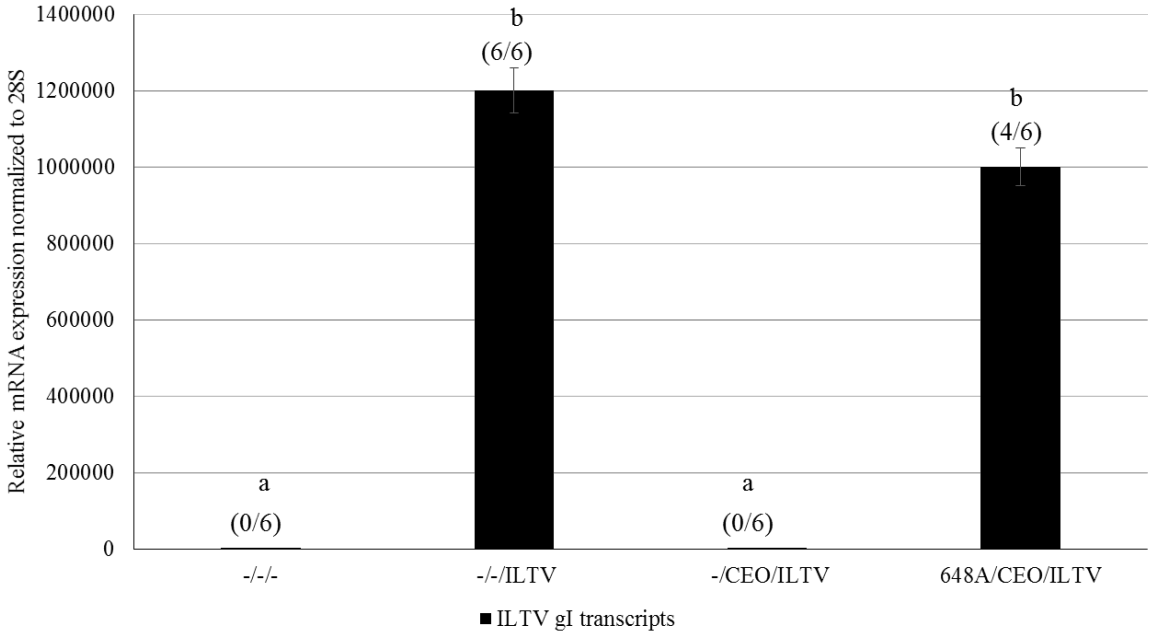

Figure 2.2 Expression of ILTV gI transcripts in tracheal exudate collected from chickens 4 days following challenge with ILTV (Experiment 2). Bar shows the average value of ILTV gI transcripts load in the positive chickens. Number in brackets above bar shows number of chickens with detectable transcripts of ILTV gI in tracheal swabs by real time RT-PCR over total number of sampled chickens. Different letters over the bars indicate that differences were statistically significant ($p<0.05$). Group -/-/- received no treatment (negative controls); Group -/-/ILTV received only ILTV challenge; Group -/CEO/ILTV received CEO vaccination and ILTV challenge and; Group 648A/CEO/ILTV infected with MDV, vaccinated with CEO and then challenged with ILTV.

Table 2.1. Development of respiratory clinical signs within six days following ILTV challenge (experiment 2)

Groups	D1[a]	D2[a]	D3[a]	D4[a]	D5[a]	D6[a]	Mortality	ITPI[b]	CS Score[b]
-/-/-	0/30 (0%)^{A}	0/30 (0%)^{A}	0/30 (0%)^{A}	0/30 (0%)^{A}	0/30 (0%)^{A}	0/30 (0%)^{A}	0	0	0^{A}
-/-/ILTV	1/25 (4%)^{A}	16/25 (64%)^{B}	19/25 (76%)^{B}	20/25 (80%)^{B}	24/25 (96%)^{B}	23/23 (100%)^{B}	2	0.56	4.36^{B}
-/CEO/ILTV	0/25 (0%)^{A}	1/25 (4%)^{A}	2/25 (8%)^{A}	0/25 (0%)^{A}	0/25 (0%)^{A}	3/25 (12%)^{A}	0	0.04	0.21^{A}
648A/CEO/ILTV	7/27 (26%)^{B}	13/27 (48%)^{B}	15/27 (56%)^{B}	19/27 (70%)^{B}	18/26 (69%)^{C}	19/26 (73%)^{C}	1	0.5	3.6^{B}

[a] Frequency of animals showing clinical signs at each day (D) post ILTV inoculation. In brackets is the percentage of chickens with ILT clinical signs

[b] ILT clinical signs were evaluate by two methods: Intratracheal pathogenicity index (ITPI) and clinical signs score (CS score). ITPI ranged from 0-2 (0= no clinical signs, 2= death). CS score ranged from 0-6 (0= no clinical signs, 6=chickens had clinical signs for 6 days).

Different superscript capital letters indicate statistically significant values ($p<0.05$).

Table 2.2. Percentage of chickens that developed ILT and/or MD gross lesions and ILT gross lesion score (experiments 1 and 2)

Exp. [a]	Group	# chickens	Frequency of chickens that developed gross lesions [b]					ILT gross lesion score [c]
			% ILT	%MD	%MD only	%ILT only	%MD and ILT	
1	-/-/-/-	30	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
1	-/-/-/ILTV	27	50^{B}	0^{A}	0^{A}	50^{B}	0^{A}	2.44^{B}
1	-/-/CEO/ILTV	29	13^{C}	0^{A}	0^{A}	13^{C}	0^{A}	1.75^{C}
1	-/648A/CEO/ILTV	27	50^{B}	74^{B}	30^{B}	11^{C}	44^{B}	2.47^{B}
1	HVT/648A/CEO/ILTV	28	70^{B}	39^{C}	10^{AB}	46^{B}	21^{B}	2.67^{B}
1	HVT+SB-1/648A/CEO/ILTV	28	63^{B}	46^{C}	14^{B}	39^{B}	32^{B}	2.63^{B}
1	CVI988/648A/CEO/ILTV	30	63^{B}	34^{C}	20^{B}	32^{B}	14^{C}	2.6^{B}
2	-/-/-/-	30	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
2	-/-/-/ILTV	25	100^{B}	0^{A}	0^{A}	100^{B}	0^{A}	3.28^{B}
2	-/-/CEO/ILTV	25	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0.58^{C}
2	-/648A/CEO/ILTV	27	41^{C}	59^{B}	31^{B}	19^{C}	28^{B}	1.85^{B}

[a] Data presented come from two experiments. Statistical analysis were done independently for each experiment. Different superscript capital letters indicate statistically significant differences ($p<0.05$).

[b] Percentage of chickens that developed gross lesions. Results are presented as the percentage of chickens with ILT (%ILT), MD lesions (%MD), only MD lesions (%MD only), only ILT lesions (%ILT only), or with both MD and ILT lesions (%MD and ILT.

[c] ILT gross lesion score is presented as the average of all chickens in each treatment group and range from 0 to 4 depending on severity of the lesions.

Table 2.3: Evaluation of lymphoid organ atrophy

Group	Relative weight[a]				Histopathology[c]				Image analysis[d]			
	Bursa		Thymus		Bursa		Thymus		Bursa		Thymus	
Replicate	1	2	1	2	1	2	1	2	1	2	1	2
-/-/-	NA	0.26^{A}	NA	0.49^{A}	No atrophy	No atrophy	No atrophy	No atrophy	139^{A}	186^{A}	134^{A}	176^{A}
-/-/ILTV	NA	0.18^{A}	NA	0.16^{B}	No atrophy	No atrophy	No atrophy	No atrophy	131^{A}	196^{A}	129^{A}	179^{A}
-/CEO/ILTV	NA	0.20^{A}	NA	0.48^{A}	No atrophy	No atrophy	No atrophy	No atrophy	135^{A}	197^{A}	121^{A}	179^{A}
648A/CEO/ILTV	NA	0.24^{A}	NA	0.40^{A}	No atrophy	No atrophy	No atrophy	No atrophy	143^{A}	193^{A}	133^{A}	176^{A}

[a] Relative weight was calculated as lymphoid organs weight (gr)/body weight (gr) multiplied by 100. Lymphoid organ weight was not measured in replicate 1. *NA = not available.

[c] Histopathology was done by assessing lymphoid organ integrity as explain in Materials and Methods.

[d] Image analysis were done with Image J (National Institute of Health, Bethesda, Maryland, USA) by calculating gray value of each pixel along a diagonal line as described in material & methods.

Different superscript capital letters indicate statistical significance ($p<0.05$).

Table 2.4: Criteria to evaluate late-MDV-IS in the ILT model[a]

Group[b]	CS score[c]	GL score[d]	ILT index[e]	Protection Index[f]	IS rank[g]
-/-/-	0	0	0		
-/-/ILTV	4.36	3.28	7.64		
-/CEO/ILTV	0.21	0.58	0.79	89.65	
648A/CEO/ILTV	3.61	1.85	5.46	28.53	68.2

[a] ILT index, protection index, and immunosuppression (IS) rank were calculated in experiment 2 using the values of clinical signs (CS) and gross lesion (GL) scores.

[c] CS score indicated the average number of days chickens showed clinical signs (CS) (values 0-6).

[d] GL score was calculated based on severity of ILT tracheal lesion (values 0-4).

[e] ILT index was calculated by adding CS score and GL score.

[f] Protection index was calculated using formula $(ILTI_{-/ILTV} - ILTI_{TXT}) / ILTI_{-/ILTV}) \times 100$. $ILTI_{-/ILTV}$ refers to ILT index of -/-/ILTV control group and $ILTI_{TXT}$ refers to ILT index of any treatment group that had been vaccinated with CEO vaccine.

[g] IS rank was calculated as $100 - ((PI_{MDV/CEO/ILTV} / PI_{CEO/ILTV}) \times 100)$.

CHAPTER 3

Efficacy of various Marek's disease vaccines protocols for prevention of Marek's disease virus-induced immunosuppression[1]

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3.1 Abstract

Marek's disease virus (MDV) induces tumors and severe immunosuppression in chickens. MDV-induced immunosuppression (MDV-IS) is very complex and difficult to study. In particular, the late MDV-IS (late-MDV-IS) is of great concern since it can occur in the absence of lymphoid organ atrophy or gross tumors. We have recently developed a model to reproduce late-MDV-IS under laboratory conditions. This model measures MDV-IS indirectly by assessing the effect of MDV infection on the efficacy of infectious laryngotracheitis (ILT) vaccination; hence the name late-MDV-IS ILT model. In this study, we have used the late-MDV-IS ILT model to evaluate if MD vaccination can protect against late-MDV-IS. One experiment was conducted to determine whether that serotype 1 MD vaccines (CVI988 and Md5-BACΔMEQ) could induce late-MDV-IS by themselves. Three additional experiments were conducted to evaluate efficacy of different MD vaccines (HVT, HVT+SB-1, CVI988, and Md5-BACΔMEQ) and different vaccine protocols (day-old vaccination, *in ovo* vaccination, and double vaccination) against late-MDV-IS. Our results show that none of the currently used vaccine protocols (HVT, HVT+SB-1, or CVI988 administered at day of age, *in ovo*, or in double vaccination protocols) protected against late-MDV-IS induced by vv+ MDV strains 648A and 686. Experimental vaccine Md5-BACΔMEQ administered subcutaneously at one day of age was the only vaccine protocol that significantly reduced late-MDV-IS induced by vv+MDV strain 686. This study demonstrates that currently used vaccine protocols confer high levels of protection against MDV-induced tumors (protection index = 100), but do not protect against late-MDV-IS; thus, commercial poultry flocks could suffer late-MDV-IS even in complete absence of

tumors. Our results suggest that MDV-IS might not be related to the development of tumors and novel control methods are needed. Further evaluation of the experimental vaccine Md5-BACΔMEQ might shed light on protective mechanisms against late-MDV-IS.

3.2 Introduction

Marek's disease (MD) is a lymphoproliferative disease of chickens of worldwide economic importance (1, 2). The etiologic agent of MD is *Gallid herpesvirus* 2 (GaHV-2) which is commonly known as Marek's disease virus (MDV) or serotype 1 MDV (3, 4). MDV is antigenically related with *Gallid herpesvirus 3* (MDV-2 or serotype 2) and *Meleagrid herpesvirus 1* (MDV-3 or serotype 3), also known as herpesvirus of turkeys (HVT). Serotypes 2 and 3 are non-oncogenic viruses isolated in chickens and turkey, respectively. Both of these replicate in chickens and can be used as vaccines (5, 6). Serotype 1 MDV includes chicken viruses that are oncogenic. Based on virulence, serotype 1 MDVs are divided into several pathotypes: mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+) (7, 8).

In the absence of vaccination, MD is a devastating disease for the poultry industry. MD vaccines were first introduced in 1968 (9). Current MD vaccines include strains of all three serotypes: Serotype 3 strains (HVT), combination of serotypes 2 strains (SB-1, 301/B) and HVT; and serotype 1 strain CVI988 (Rispens), 1 which is the most effective vaccine currently used against vv+ MDV strains (5, 6, 10-15). MD vaccines have been very successful in controlling MDV-induced tumors. However, in spite of the success of MD vaccines, MD is still a problem for the poultry industry due to the continuous evolution of

MDV towards more virulence. Newly emergent MDV strains are able to break vaccine immunity and also they induce a variety of syndromes other than tumors, among which MDV-induced immunosuppression (MDV-IS) is the one of greatest concern (16, 17).

Control of newly emergent, highly virulent MDV strains has required the use of various vaccination strategies such as *in ovo* vaccination, protective synergism, revaccination, adjuvants, and recombinant vaccines. *In ovo* vaccination is used in broilers and broiler breeders in the USA and its use is becoming more popular in other countries. MD vaccine is injected into embryos at 18 days of embryonation by the amniotic route. *In ovo* vaccination allows administration of vaccines three days before chickens can get exposed to MDV, reduces labor, and hastens the development of the chicken immune system (18, 19). Numerous studies have demonstrated that *in ovo* administration of MD vaccines results in greater protection against MDV-induced tumors than vaccination at day of age (20-22). Another strategy to increase vaccine efficacy is the use of two or more MDV strains of various serotypes in the same vaccine. It has been demonstrated that combination of serotypes 2 and 3 strains results in protective synergism and the combination of vaccines provide better protection than individual vaccine used alone (23, 24). The mechanisms involved in protective synergism are poorly understood and it only occur between strains of serotypes 2 and 3 but not between strains of serotypes 1 and 3 (14, 25). Revaccination can also enhance vaccination efficacy when properly conducted (26-28). The positive effect of revaccination has been reproduced under laboratory conditions when both vaccines are administered before challenge and the second vaccine is more protective than the first vaccine administered (27). Gimeno et al. have shown that revaccination is most efficacious

when the first vaccine was administered *in ovo* and the second vaccine was administered at day of age (27). In the last decade, highly efficient recombinant serotype 1 MD vaccines have been developed (29-31). Deletion of oncogene meq from vvMDV Md5 (Md5-BACΔMEQ) strain resulted in a vaccine that provide better protection than CVI988 when challenged against vv+ strains at day of age (32, 33). However, this strain induces severe lymphoid organ atrophy in maternal antibody negative chickens and cannot be licensed under current regulations (32).

MD vaccines protect against the development of tumors as well as against the development of other syndromes such as transient paralysis, lymphodegenerative syndromes and lymphoid organ atrophy, and arteriosclerosis. However, MD vaccines do not protect against superinfection and transmission of MDV. Preliminary data suggest that one-day-old vaccination might not protect against late-MDV-IS (34). MDV-IS can be divided into two phases, early immunosuppression associated with early cytolytic infection of lymphoid organs and late immunosuppression that occur during the establishment of latency and tumor development (35). Early-MDV-IS is controlled by MAb against MDV and vaccination and it is not considered to have any relevance under commercial conditions (36, 37). Late-MDV-IS could occur in commercial flocks (34) and is a threat to the poultry industry. In a previous work, we have developed a model to study late-MDV-IS under laboratory conditions. This model indirectly evaluates late-MDV-IS by assessing the effect of vv+MDV infection at day of age on the efficacy of infectious laryngotracheitis (ILT) vaccination at 15 days of age followed by challenge exposure with infectious laryngotracheitis virus (ILTV) at 30 days of age. Our studies have shown that late-MDV-IS can happen in commercial chickens bearing

maternal antibody against MDV that did not have lymphoid organ atrophy or gross tumors. Late-MDV-IS markedly decreased the efficacy of ILT vaccination (34) and could affect the efficacy of vaccination programs against other diseases as well. It is unknown if MD vaccination protocols that confer high protection against the development of tumors also could protect against late-MDV-IS. Moreover, it is unknown if serotype 1 MD vaccines that induces lymphoid organ atrophy in maternal antibody chickens (i.e. Md5-BACΔMEQ) can induce late-MDV-IS in the ILT model.

The objectives of this study were to 1) determine if serotype 1 MD vaccines can induce late-MDV-IS and 2) evaluate if MD vaccination protocols that protect against MDV-induced tumors also protect against late-MDV-IS.

3.3 Materials and Methods

Chickens. Commercial specific pathogen free SPAFAS chickens (Charles River SPAFAS, N Franklin, CT) were used as MDV shedder chickens. Female commercial meat type chickens (grandparents) bearing MAb against MDV were used as experimental chickens Experimental chickens came from dams that were vaccinated with vaccines of the three serotypes (HVT, SB-1, and CVI988).

Viruses and vaccines. Serotype 1 MDV strains, 648A (vv+) at passage 12 in chicken embryo fibroblast (CEF) and 686 (vv+) at passage 10 in duck embryo fibroblast (DEF) with one additional passage in chicken kidney cells (CKC) were used as MDV challenge (8). Commercial strains HVT (6, 12), bivalent HVT-SB1 (5, 14, 24, 38), and CVI988 (10) from two different manufacturers (A and B) were used for vaccine trial. Differences in protection

between CVI988 A and B against early challenge with a vv+MDV have been reported (39, 40). Experimental vaccine, Md5-BACΔMEQ (passage 5 in chicken embryo fibroblast) was also used (41). Chicken embryo origin (CEO) vaccine strain of ILTV was obtained from a commercial manufacturer (Merial Select, Inc., Gainesville, GA, USA). Illinois-N71851 strain of ILTV that has been characterized as a virulent ILTV strain were used for ILT challenge (42).

Real time PCR. DNA was extracted from feather pulp and spleen using ArchivePure DNA tissue kit (5-Prime Inc, Gaithersburg, Maryland, USA) following manufacturer's recommendations.

DNA samples were amplified with three pairs of primers specific for the glycoprotein B (gB) gene of serotype 1 MDV, MDV EcoRI Q fragment of serotype 1 MDV strains (Meq) and for the chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The sequence for the respective forward and reverse primers are listed in Table 3.2. Amplifications were done using an Mx3005 (Stratagene, La Jolla, CA) in a 25-ul PCR reaction containing 50 ng of DNA, 0.2 μM of each primer, and SYBR® Green PCR master mix (Brilliant® SYBR® Green, Biocrest-Stratagene, Cedar Creek, TX). The reaction was cycled 50 times at 95°C denaturation for 15 sec and a 60°C combined annealing/extension for 60 sec. Fluorescence was acquired at the end of the annealing/extension phase. The melting curves were obtained at the end of amplification by cooling the sample at 2.0°C/sec to 60°C and then increasing the temperature to 95°C at 0.1°C/sec. The parameter threshold cycle (Ct) was calculated for each PCR reaction by establishing a fixed threshold. Ct is

defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Relative quantification of the amount of target in unknown samples was accomplished by the comparative Ct method. Ct ratios were established for each sample (Ct ratio GAPDH-gB = Ct GAPDH/Ct gB). The higher the Ct ratio, the higher the load of MDV (43, 44).

Real time RT-PCR. RNA was extracted from spleen samples using the Perfect Pure RNA tissue kit (5-Prime Inc, Gaithersburg, Maryland, USA) following manufacturer's recommendation. Real time reverse transcriptase PCR (real time RT-PCR) was performed to measure the transcription of MDV ICP4, pp38, gB and Meq genes. Primers sequence are listed in Table 3.2. Housekeeping gene 28S rRNA were used as internal control (45). Amplifications were done in an Mx3005 Stratagene (Stratagene, La Jolla, California, USA) thermocycler by amplifying 25µl reactions using Brilliant II SYBR® Green Q-PCR Master Mix (Agilent Technologies, Santa Clara, CA, USA). The profile cycles used were 1 cycle of 50°C for 30 min; 1 cycle of 95°C for 10 min; 50 cycles of 95°C for 10 sec and 56°C for 1 min; and 1 cycle of 95°C for 1 min, 55°C for 30 sec, and 95°C for 30 sec. The melting curves were obtained at the end of amplification by cooling the sample at 2.0^{o}C/s to 60^{o}C and then increasing the temperature to 95^{o}C at 0.1^{o}C/s. Samples were ran in duplicates and the Ct was averaged. Average Ct value were subtracted from total number of cycles (50 - average Ct). Correction for differences in RNA levels between samples was done by using a 28S correction factor via the formula: (50 - overall mean Ct 28S rRNA-specific product from all samples) / (50 - average Ct for 28S rRNA-specific product for each sample). Corrected viral

gene means was calculated using the formula: 28S correction factor X (50 - Ct value for viral gene) X viral gene slope/28S gene slope) (46).

Analysis of microRNA expression by real-time PCR. First strand complementary DNA (cDNA) was synthesized from 1μg of polyadenylated total RNA from each spleen samples using a miRNA 1st strand cDNA synthesis kit (Agilent, Santa Clara, CA) following the manufacturer's instructions. For miRNA real time PCR, each reaction contained 1 μl of cDNA and 24 μl High-specificity miRNA QPCR Core Reagent Kit Master Mix (Agilent Technologies, Santa Clara, CA, USA). PCR was performed using the following conditions: 95°C for 15 min followed by 40 cycles of [95°C 10 sec and 60°C 20 sec] using Mx3005 Stratagene (Stratagene, La Jolla, California, USA) thermocycler. Expression of MDV miRNA mdv1-miR-M4-5p and chicken snoU83B was conducted using forward primers previously reported and they are listed in Table 3.2 (47). Average Ct value were subtracted from total number of cycles (40 - average Ct). Correction for differences in miRNA levels between samples was done by using a snoU83B correction factor via the formula: (40 - overall mean Ct snoU83B miRNA-specific product from all samples) / (40 - average Ct for snoU83B miRNA product for each sample). Corrected viral gene means was calculated using the formula: snoU83B correction factor X (40 Ct value for viral gene) X viral gene slope/ snoU83B gene slope).

Serology. Serum samples collected in Experiment 3 were analyzed for antibody titers against ILTV using the commercial ProFLOK® Fowl Laryngotracheitis Virus Antibody Test Kit

(Synbiotic Corporation, Kansas City, MO). The test was performed following the manufacturer's recommendations.

Experimental Design. Animal experiments were conducted following the guidance and under approval of North Carolina State University Institutional Animal Care & Use Committee (IACUC). Three experiments were conducted using the experimental design of the late-MDV-IS ILT model as reported (34). Details of the model are shown in Figure 3.1. Briefly, shedder chickens were vaccinated with HVT at 18 days of embryonation to protect chickens against transient paralysis and ensure survival. At hatch, shedder chickens were infected with 500 PFU of vv+ strain MDV 648A (experiments 1 and 2) or 686 (experiment 3) strain subcutaneously and maintained in isolation for 15 days prior to beginning of experiment. Experimental chickens housed with the shedders were tested by real time PCR for both MDV and HVT at 7 days and at the end of the experiment. Horizontal transmission of MDV was confirmed by detecting MDV DNA in the feather pulp of experimental chickens at 7 days of age. Horizontal transmission of HVT from the shedders to the experimental chickens did not occur in any of the experiments of this study.

In each experiment, newly hatched meat type experimental chickens were wing banded, divided into groups (42 chickens per group) and placed in environmentally controlled room (BSL-2) for the rest of the experiment. Details of each experiment are shown in Table 3.1. Each experiment included four control groups, 1) Group 1 received no treatment (negative controls); 2) Group 2 was challenged with ILTV; 3) Group 3 was vaccinated with CEO and challenged with ILTV; and 4) Group 4 was infected with MDV

(648A or 686) by contact exposure at day of age, vaccinated with CEO and challenged with ILTV. In addition, each experiment included one or more MD-vaccinated groups to evaluate if MD vaccines can induce late-MDV-IS (experiment 1) and to determine whether currently available vaccine protocols could protect against late-MDV-IS (experiments 2 and 3). All MD vaccinated chickens were vaccinated with CEO and challenged with LTV. Experiment 1 included two groups that received a serotype 1 MD vaccine (CVI988-A or Md5-BACΔMEQ) but were not challenged with vv+ strain 648A. Experiment 2 included three groups that were vaccinated at day of age with a MD vaccine (HVT, HVT+SB1, CVI988-A) and then were challenged with vv+MDV strain 648A. Experiment 3 included a group that was vaccinated against MD *in ovo* (HVT +CVI988-B), a group that received a revaccination protocol (HVT *in ovo* and CVI988-B at day of age), and a group that was vaccinated at day of age with experimental vaccine Md5-BACΔMEQ. I*n ovo* vaccination was conducted manually at 18 days of embryonation via intra-amniotic route as previously described (22).

When meat type chickens were 15 days of age, they were vaccinated with CEO vaccine via drinking water per manufacturer's recommendations. Two weeks later (day 30 of the experiment), chickens were challenged intratracheally with 2000 PFU of ILTV. At this time all chickens were identified with colored leg bands. Every chicken was monitored daily for six days between challenge with ILTV and the termination of the experiment to evaluate if clinical signs of ILT were present. All chickens were necropsied at termination (day 7 after ILTV challenge) or at time of death, and evaluated for gross lesions consistent with ILT and MD.

In experiment 3, feather samples and blood were collected from every chicken at day 6 post ILTV for DNA extraction and serology, respectively. In addition, spleen samples from six chickens per treatment group were collected for nucleic acid extraction.

Clinical Signs. Each bird was identified and observed daily for clinical signs of ILT such as gasping, coughing, sneezing, conjunctivitis and expectoration. Mortality was also recorded.

Development of ILT clinical sings (CS) was assessed by ILT-CS score as previously described (34). Briefly, ILT-CS score was calculated as the total number of days a particular chicken showed clinical signs (range from 0 to 6).

Gross Lesions. Upon necropsy, dead birds were evaluated for ILT and MD lesions. ILT gross lesions (GL) were scored from 0 to 4 as follow: 0 = normal, 1 = presence of light mucus, 2 = congested, thick mucus or bloody mucus, 3 = presence of caseous exudate, and 4 = tracheal plugs.

MD lesions were observed by evaluating for presence of tumors in nerves, visceral organs, eye and skin.

Assessment of late-MDV-IS. Evaluation of late-MDV-IS was done based on ILT clinical signs (CS) and gross lesions (GL) as previously described (34). Number of days exhibiting CS was calculated for each chicken (values 0-6). GL in trachea at termination were scored based on severity (values 0-4). ILT index was calculated to assess severity of the disease based on CS and GL.

ILT index (ILTI) = CS + GL (values 0-10)

Protection index of the CEO vaccine (PI CEO) was calculated using ILTI values from the positive control group (-/ILTV) and the CEO vaccinated groups (-/CEO/ILTV, and MDV/CEO/ILTV) to measure protection provided by CEO vaccine.

$$PI\ CEO = (ILTI_{-/ILTV} - ILTI_{TXT}) / ILTI_{-/ILTV}) \times 100$$

$ILTI_{-/ILTV}$ is ILT index of -/-/ILTV control group.

$ILTI_{TXT}$ is ILT index of any treatment group that had been vaccinated with CEO vaccine.

Results are presented as PI CEO of the MDV challenged groups (MD vaccinated or unvaccinated) relative to the value of the PI CEO of the control group (None/None/CEO/ILTV).

Assessment of MDV-induced tumors. MDV-induced tumors were evaluated by gross inspection during necropsy at the termination of the experiment or when chickens died. In addition, early diagnosis using real time PCR was conducted as described (43, 48, 49). Briefly, oncogenic MDV DNA load was evaluated by real time PCR in feather pulp from every chicken at the termination of the experiment. Ct ratio was calculated for each sample by dividing the Ct value of housekeeping gene (GAPDH) by the Ct value of the MDV gene (gB) amplified. Based on the Ct ratio, sample were classified as negative (GAPDH gene amplified but the MDV gene did not); latently infected (Ct ratio is lower than the threshold level 1.7); neoplastically transformed (Ct ratio is equal or higher than the threshold level 1.7).

To evaluate the efficacy of MD vaccines to protect against the development of tumors two protection indexes were calculated, one based on gross tumors (PI MD) and other based on oncogenic MDV DNA load (PI MDV DNA) as described (40, 50).

PI MD = (% MD $_{-/MDV/CEO/ILTV}$ – %MD $_{MD\ vaccine/MDV/CEO/ILTV}$) / % MD $_{-/MDV/CEO/ILTV}$) x 100.

PI MDV DNA = (PIMDV DNA $_{-/MDV/CEO/ILTV}$ – PI MDV-DNA $_{MD\ vaccine/MDV/CEO/ILTV}$) / PI MDV DNA $_{-/MDV/CEO/ILTV}$) x 100.

Statistical analysis. Data were analyzed with statistical programs Statistica (Stat Soft, Tulsa, Oklahoma, USA) and SPSS (IBM, New York, USA). Comparison between two groups were conducted using Student's t test while comparison between more than two groups were conducted using one-way analysis of variance (ANOVA) test. The Scheffe test was used as a post hoc analysis. In experiment 3, correlation between PI CEO with PI MD and PI MDV DNA was evaluated by Pearson's test using the STATISTICA™ software. The level of significance was $p<0.05$.

3.4 Results

Serotype 1 MD vaccines did not induce late-MDV-IS (Experiment 1). The immunosuppressive ability of serotype 1 MD vaccines (CVI988 and Md5-BACΔMEQ) was evaluated (Figure 3.2). PI CEO was calculated to evaluate the ability of CEO to protect against challenge with ILTV in each group. Data of each group is presented relative to the PI CEO of the control group None/None/CEO/ILTV (baseline of 100%). PI CEO of group None/648/CEO/ILTV was significantly lower (31.8%) than that of the control group

None/None/CEO/ILTV (100%). No significant differences were found between groups that received serotype 1 MD vaccines (CVI988/None/CEO/ILTV, and Md5-BACΔMEQ/None/CEO/ILTV) and the control group None/CEO/ILTV (86.4% and 84.4% vs 100%).

MD vaccines did not protect against late-MDV-IS when administered at 1-day-old (Experiment 2). Three commercial vaccines (HVT, HVT-SB1 and CVI988-A) were administered at 1-day-old via subcutaneous route following manufacturer's recommendations. Results are presented in Figure 3.3. Data of each group is presented relative to the PI CEO of the control group None/None/CEO/ILTV (baseline of 100%). The PI CEO of all groups challenged with 648A regardless of vaccination status (PI CEO $_{\text{None/648A/CEO/ILTV}}$ = 32%, PI CEO $_{\text{HVT/648A/CEO/ILTV}}$ = 76.5%, PI CEO $_{\text{HVT+SB-1/648A/CEO/ILTV}}$ = 42%, PI CEO $_{\text{CVI988/648A/CEO/ILTV}}$ = 35.4%) was significantly lower than the PI CEO in the control group None/None/CEO/ILTV (100%).

Ability of highly effective MD vaccination protocols against MDV-induced tumors to protect against late-MDV-IS (Experiment 3). The most effective protocols known against MDV-induced tumors (*in ovo* CVI988-B+HVT; *in ovo* HVT with revaccination at 1-day with CVI988-B, and experimental vaccine Md5-BACΔMEQ at day of age) were evaluated for their ability to protect against late-MDV-IS. Results are shown in Figure 3.4. Data of each group is presented relative to the PI CEO of the control group None/None/CEO/ILTV (baseline of 100%). PI CEO of groups None/686/CEO/ILTV, HVT(IO)-CVI988-B(D1)/686/CEO/ILTV and HVT+CVI988-B(IO)/686/CEO/ILTV were significantly lower

(45.6%, 53.9%, and 54.8%, respectively) than PI CEO of control group None/None/CEO/ILTV (100%) and of group Md5-BACΔMEQ/686/CEO/ILTV (95.2%). There were no significant differences between PI CEO of control group None/None/CEO/ILTV and PI CEO of Md5-BACΔMEQ/686/CEO/ILTV group.

Evaluation of different vaccine protocols on antibody titers against ILT (Experiment 3). Serum collected at 6 days post ILTV infection was evaluated by ELISA for antibody titers against ILTV (Figure 3.5) Chickens in CEO vaccinated control group (-/-/CEO/ILTV) showed highest level of antibody titer (3060). Antibody titer in MDV infected control group (-/686/CEO/ILTV), revaccination group (HVT (IO)-CVI988-B (D1)/686/CEO/ILTV) and bivalent in ovo group (HVT+CVI988 -B(IO)/686/CEO/ILTV) was significantly lower (1132, 1182, and 1594 respectively) compared to CEO vaccinated control group (-/-/CEO/ILTV) Chickens vaccinated with Md5-BACΔMEQ in Md5-BACΔMEQ/686/CEO/ILTV group shows no significant difference in antibody titer (2839) compared to CEO vaccinated control group -/-/CEO/ILTV (3060).

Protection of MD vaccines against tumors and correlation of MDV-induced tumors with late-MDV-IS (Experiments 2 and 3). MDV-induced tumors were evaluated during necropsy in all experiments. In experiment, 3 development of MD tumors was also evaluated by measuring MDV DNA load in feather pulp at the termination of the experiment (7 days post ILTV challenge). The level of protection of various MD vaccines protocols against MDV-induced tumors (evaluated by gross inspection and viral DNA load) is presented in Table 3.3. Results show that vaccination at day old provided variable protection against MD

gross tumor development (PI MD values of 52 for HVT, 92 for HVT+SB1 and 74 for CVI988). The use of the most efficient vaccine protocols against MD tumors (*in ovo*, revaccination and Md5-BACΔMEQ) provided high level of protection against MDV-induced tumors evaluated by gross inspection (PI MD = 100 in all vaccinated groups) and by viral DNA load (PI MDV DNA values of 91.3, 95.5 and 98.9 for in ovo, revaccination, and Md5-BACΔMEQ, respectively). These results occurred despite poor protection against late-MDV-IS (PI CEO value of 51.3 in *in-ovo* vaccinated group and 52.2 in the revaccinated group).

Correlation analysis was conducted for PI MD gross tumors and PI MDV viral load; PI MD gross tumors and PI CEO; and PI DNA MDV viral load and PI CEO in Experiment 3. Correlation analysis shows significant high correlation (r=0.99) between PI MD gross tumors and PI MDV DNA load. However, no correlation ($p > 0.05$) was found between MDV-induced tumors, evaluated as gross tumors (PI MD) or as load of MDV DNA (PI MDV DNA) and MDV-IS (evaluated as PI CEO).

MDV replication in spleen of chickens following various MD vaccine protocols (Experiment 3). Detection of oncogenic MDV DNA as well as expression of various MDV transcripts were evaluated in the spleen of 6 chickens per treatment group in experiment 3 at the termination of the experiment (37 days of age). Results are presented in Table 3.4. MDV DNA (gB and Meq) was detected in 100% of the spleens (6 out of 6) of all challenged groups. Transcription of all evaluated virus genes and mdv1-miR-M4-5p load were statistically significantly higher in the unvaccinated challenged group (None/686/CEO/ILTV) than in all MD vaccinated groups. No significant differences ($p<0.05$) were found in the viral

gene transcriptions between the groups that received *in ovo* vaccination (HVT+CVI988 -B(IO)/686/CEO/ILTV) and the revaccination protocol groups (HVT (IO)-CVI988-B (D1)/686/CEO/ILTV). However differences were found in the transcription of meq between the group Md5-BACΔMEQ/686/CEO/ILTV and the other two vaccinated groups (HVT+CVI988 -B(IO)/686/CEO/ILTV and HVT (IO)-CVI988-B (D1)/686/CEO/ILTV). Even though oncogenic MDV DNA was detected in the spleens of all chickens in group Md5-BACΔMEQ/686/CEO/ILTV, no transcripts of meq was detected. Similarly, no mdv1-miR-M4-5p was detected in the spleens of group Md5-BACΔMEQ/686/CEO/ILTV.

3.5 Discussion

MDV-induced tumors, the best studied feature of MDV infection, can be effectively controlled by vaccination (51). Recently we have demonstrated that in addition to tumors, vv+MDV strains can induce late-MDV-IS in commercial meat type chickens bearing MAb against MDV (34). In this study, we have demonstrated that none of the currently used vaccination protocols against MD, even the most efficacious, can protect against late-MDV-IS induced by vv+MDV strains 648A and 686. On the other hand, our study shows that neither CVI988 nor experimental serotype 1 MD vaccine Md5-BACΔMEQ induce late-MDV-IS. Experimental vaccine Md5-BACΔMEQ induces severe lymphoid organ atrophy (early-MDV-IS) in chickens lacking MAb (52). Our results demonstrate that when using commercial chickens bearing MAb, Md5-BACΔMEQ not only did not induce late-MDV-IS but it was the only vaccine able to protect against both MDV-induced tumors and late-MDV-IS.

MDV induces two phase of immunosuppression. The first phase of immunosuppression (early MDV-IS) is associated with the replication of MDV in the lymphoid organs, destruction of lymphocytes and subsequent lymphoid organ atrophy. The second phase of immunosuppression (late-MDV-IS) occurs after MDV undergoes latency and persists for an unknown period of time (53). Early MDV-IS is easily controlled by the combination of MAb against MDV and vaccination, thus early-MDV-IS is not considered to have any relevance in commercial poultry (36, 37). Late MDV-IS is more complex and poorly understood. In previous studies, we have developed a model to reproduce late-MDV-IS under laboratory conditions (late-MDV-IS ILT model) (34). This model indirectly evaluates late-MDV-IS by assessing the effect of early infection with a vv+ MDV strain on the efficacy of ILT vaccines. In the present study we have confirmed the validity of this model and demonstrated that vv+MDV not only affects cellular immunity, necessary for CEO protection (54), but also humoral immune responses. Furthermore, we have demonstrated that vaccination protocols that protect against tumors do not protect against late-MDV-IS. This finding confirms that MDV-IS is even more complex than first thought. There is an early-MDV-IS associated with replication in lymphoid organs in chickens lacking MAb. There is also a late-MDV-IS evaluated in this study that seems to be unrelated to tumors. Finally, there also might be another form of late-MDV-IS that is associated with tumors since neoplastic cells have been shown to be capable of inducing immunosuppression (55-57). The late-MDV-IS ILT model can be very useful to further evaluate mechanisms behind the late-MDV-IS that is not related to tumors; this form of MDV-IS cannot be

controlled by the current vaccination procedures and is difficult to diagnose in commercial flocks.

The immunosuppressive ability of MD vaccines has been controversial. Vaccines of all three serotypes have been found to induce minor changes in the lymphoid organs in chickens lacking MAb (58, 59). Islam et al (60) reported that HVT induced mild immunosuppression since it reduced circulating B- and T-cells although it was not associated with lymphoid organ atrophy or decreased humoral antibody responses. On the other hand, Reddy et al (61) showed that HVT did not affect specific lymphoproliferative responses towards tetanus toxoid in chickens. Our results show that neither CVI988 nor experimental vaccine Md5-BACΔMEQ induced late-MDV-IS when administered at day of age. Experimental vaccine Md5-BACΔMEQ was developed by deleting *meq* gene from the very virulent Md5 MDV strain genome. The initial recombinant vaccine was made using overlapping cosmids and was named as rMd5ΔMeq (31). Later, using Md5-BAC technology a similar strain was developed that was named Md5-BACΔMEQ (29) and it is the one used in this study. Both rMd5ΔMeq and Md5-BACΔMEQ provided better protection than commercial CVI988 vaccines following early challenge with vv+ MDV (29, 32, 33, 62). However, both of them (rMd5ΔMeq and Md5-BACΔMEQ) induced severe early-MDV-IS in chickens lacking MAb against MDV (52). Attenuation in cell culture (up to 40^{th} passages) can reduce the ability to induce lymphoid organ atrophy but it also decreases protection (63). The ability of meq deleted vaccines to induce early-MDV-IS has hampered any possibility of licensing and to date they cannot be used in commercial poultry. The results of this study,

however, shows that administration of Md5-BACΔMEQ to one-day-old commercial meat type chickens not only did not induce late-MDV-IS but it was the only vaccine protocol able to protect against it. Since early-MDV-IS has no relevance in commercial flocks but late-MDV-IS might have an enormous economic impact on the poultry industry, the safety and potential use of meq deleted vaccines should be reevaluated.

To our knowledge this is the first study that show that protection against MDV-induced tumors is not enough to protect against late-MDV-IS. The relevance of these findings affect both our understanding of the pathogenesis of MD and the impact that late-MDV-IS might have in commercial flocks. It has been generally considered that late-MDV-IS is mainly due to the development of tumors. In this study, we have demonstrated that chickens properly protected against tumors still developed late-MDV-IS; and therefore late-MDV-IS evaluated in this model might be unrelated to the development of tumors. Further studies need to be conducted to further evaluate the mechanism behind late-MDV-IS. On the other hand, our findings show that none of the currently available protocols to protect against MD (even the most protective) can protect against late-MDV-IS. Therefore, commercial chickens that are exposed to vv+MDVs during the first days of life likely will experience late-MDV-IS even if they do not develop other manifestations of MD. The economic consequences of this findings are significant since late-MDV-IS can compromise both cellular and humoral immune responses.

Differences found between Md5-BACΔMEQ and other MD vaccines used in this study suggest that there might be differences in the protective mechanisms among them.

Traditionally used vaccines (HVT, SB-1, CVI988) protected against tumors and reduced the transcription of oncogenic MDV genes. However Md5-BACΔMEQ not only reduced transcription of other MDV genes (ICP4 and pp38) but it blocked completely transcription of meq and expression of mdv1-miR-M4-5p. Lee et al. reported that Md5-BACΔMEQ reduced oncogenic MDV load by limiting its replication (33). Our results confirm those findings and suggest that Md5-BACΔMEQ blocks expression of genes that are necessary for the oncogenic MDV to reactivate and for the development of tumors and late-MDV-IS. Meq is a 339 amino acid protein with a N-terminal bZIP domain and proline rich C-terminal transactivation domain (64). Several studies have shown that Meq is an MDV oncogene (31, 65-67) and it is highly expressed in tumors (64). mdv1-miR-M4 is a well characterized miRNA of MDV-1 that shares the same seed sequence with chicken gga-miR-155 and also with Kaposi's sarcoma herpesvirus microRNA kshv-miR-K12-11 (68-70). Mir-M4-5p was identified to be highly expressed in tumors (70) and it is involved but not essential in MDV induced tumorigenesis (71, 72). On the other hand, Morgan et al suggested that mdv1-miR-M4 might be involved in MDV-IS (73). Further studies are warranted to further evaluate the role of meq and mdv1-miR-M4 on late-MDV-IS.

MD is a very complex disease that includes a variety of syndromes of which tumors have the largest economic impact for the poultry industry. Today, we have efficient vaccine protocols to successfully protect against MDV-induced tumors. However, newly emergent MDVs has acquired additional pathogenic features that results in the development of new syndromes such as late-MDV-IS. In the present study we have demonstrated that currently used vaccination programs are insufficient to protect against late-MDV-IS; and this new

syndrome induced by vv+MDVs could have devastating effects on the poultry industry. Our results suggest that mechanisms of late-MDV-IS are unrelated to tumors and show that an experimental vaccine (Md5-BACΔMEQ) can protect against it. The late-MDV-IS model that we developed in previous work (34) is a suitable tool to study late-MDV-IS and could be used to better understand mechanisms of MDV-IS and develop methods of diagnosis and control.

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3.7 Figures and tables

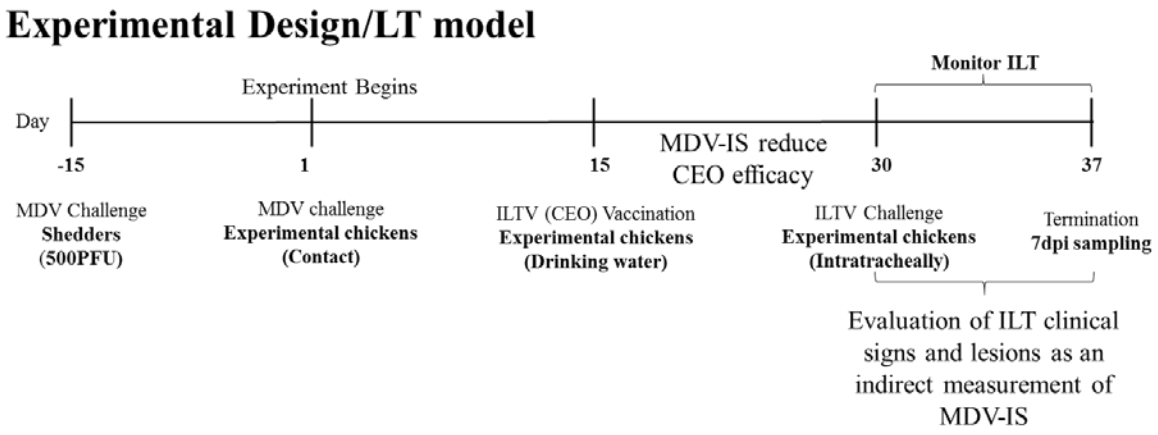

Figure 3.1. Timeline of the late-MDV-IS ILT model. Shedder chickens hatched 15 days before the experiment started and they were challenged with 500 PFU of MDV via subcutaneous at day of age. Two weeks later, once shedder chickens start transmitting MDV, they were comingled with the experimental 1 day-old meat type chickens. Experimental chickens were vaccinated against MD either in ovo or at day of age or they could remain unvaccinated. MDV challenged occurred by contact with the shedders. When experimental chickens were 15 days of age, they were vaccinated against ILT with the modified alive vaccine CEO via drinking water. Two weeks later, chickens were challenged with ILTV via intratracheally. Development of ILT was evaluated by daily inspection of clinical signs in each experimental chicken and by gross inspection of tracheal lesions at the termination of the study. MDV-IS was evaluated as the ability of MDV to reduce the efficacy of CEO vaccination.

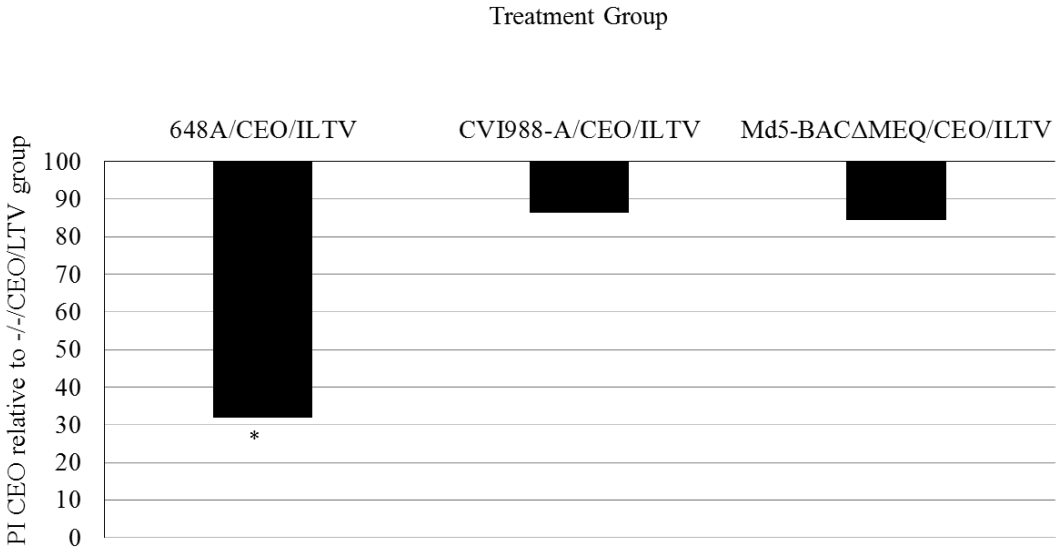

Figure 3.2 (Experiment 1). Serotype 1 MD vaccines did not induce late-Marek's disease virus-induced immunosuppression (late-MDV-IS). The efficacy of CEO vaccine against challenge with ILTV was evaluated in groups that were previously infected with vv+MDV strain 648A or with a serotype 1 MD vaccine (CVI988-A, Md5-BACΔMEQ). Results are presented as the protective index (PI CEO) relative to the control group -/CEO/ILTV. Asterisk (*) under the bars indicate that differences were statistically significant ($p<0.05$) compared to -/-/CEO/ILTV control group.

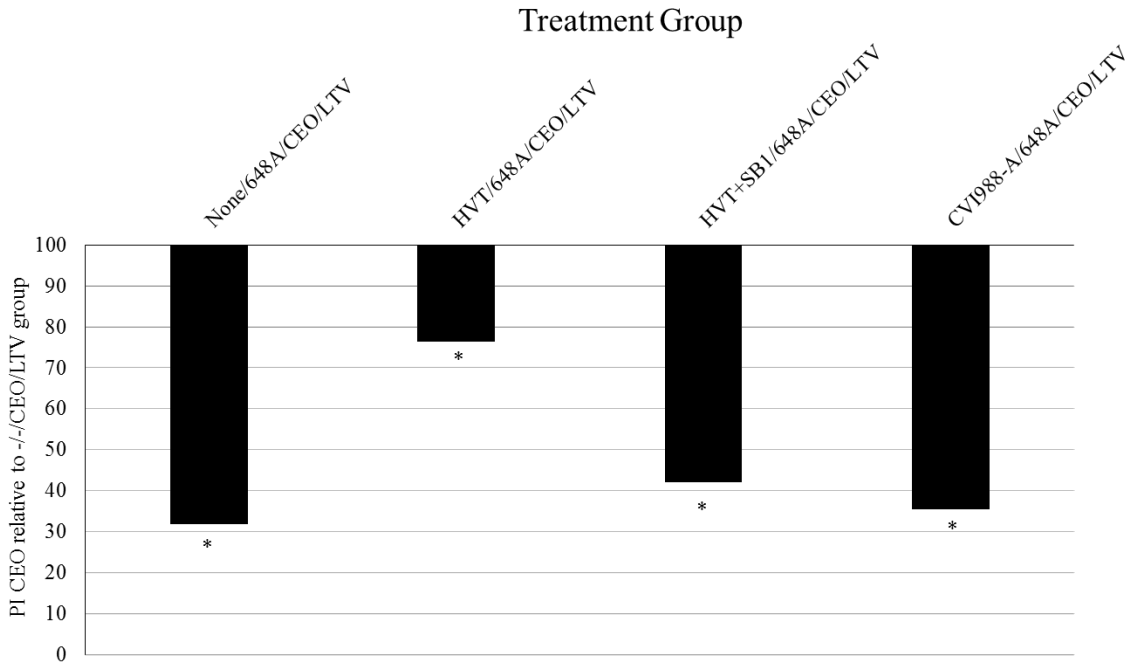

Figure 3.3 (Experiment 2). Efficacy of MD vaccines administered at day of age against late-MDV-IS. The efficacy of CEO vaccine against challenge with ILTV was evaluated in groups that were previously infected with vv+MDV strain 648A and there were either unvaccinated or vaccinated against MD (HVT, HVT+SB-1, and CVI988-B) at day of age. Results are presented as the protective index (PI CEO) relative to the control group -/CEO/ILTV of their corresponding experiment. Asterisk (*) under the bars indicate that differences were statistically significant ($p<0.05$) compared to -/-/CEO/ILTV control group.

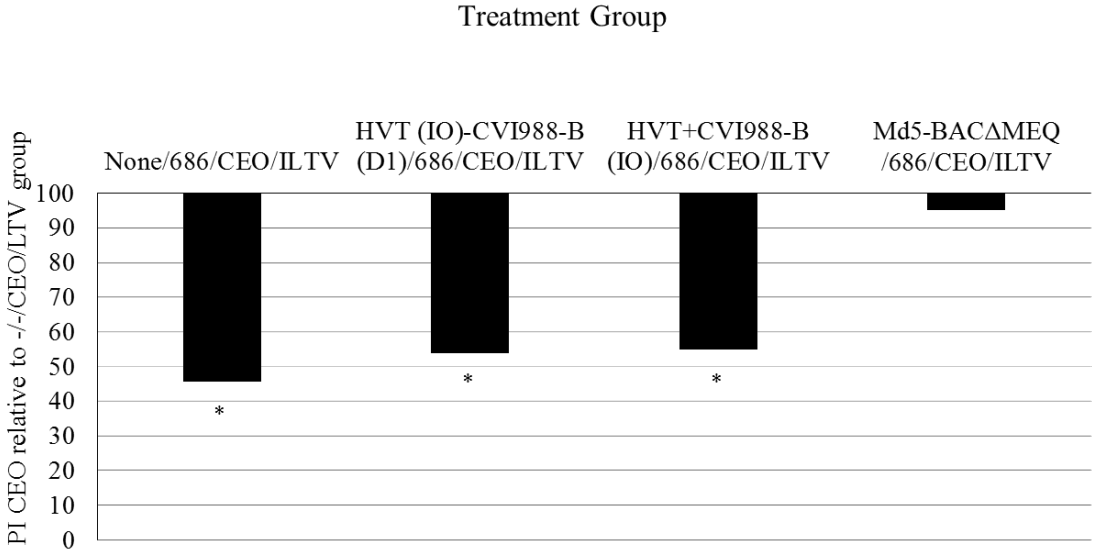

Figure 3.4 (Experiment 3). Ability of various MD vaccine protocols, highly protective against MDV-induced tumors, to protect against late-MDV-IS. The efficacy of CEO vaccine against challenge with ILTV was evaluated in groups that were previously infected with vv+MDV strain 686 and there were either unvaccinated or vaccinated against MD (HVT +CVI988-B in ovo, HVT in ovo + CVI988-B at day of age, or Md5-BACΔMEQ at day of age). Results are presented as the protective index (PI CEO) relative to the control group -/CEO/ILTV. Asterisk (*) under the bars indicate that differences were statistically significant ($p<0.05$) compared to -/-/CEO/ILTV control group.

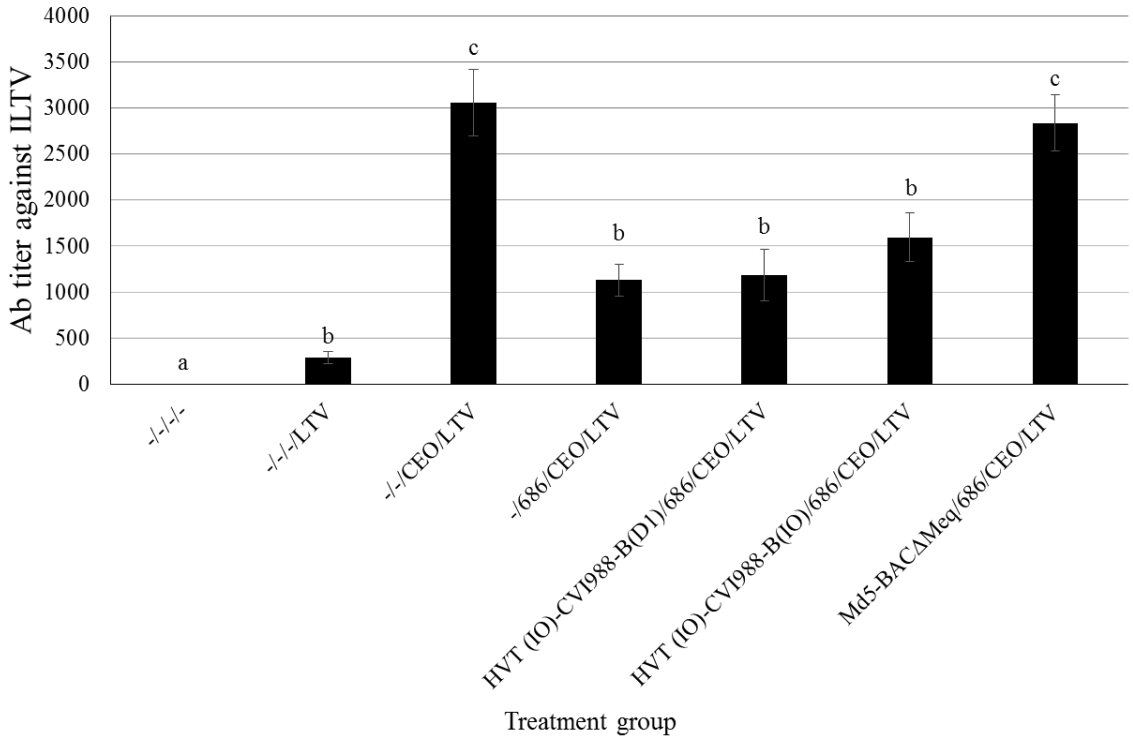

Figure 3.5 (Experiment 3). ILT virus-specific antibody titers determined using commercial ELISA kit. The extension of protection of MD vaccine on humoral immunosuppression were evaluated in Experiment 3. Values identified by different letters are statistically significant ($p<0.05$).

Table 3.1. Experimental design

	MD vaccination and challenge				ILTV vaccination and challenge			
Exp[a]	**Vaccine[b]**	**Dose /Age[b]**	**Challenge[c]**	**Route/Age[c]**	**Vaccine[d]**	**Dose/Age[d]**	**Challenge[e]**	**Dose/Age[e]**
1,2,3	-	-	-	-	-	-	-	-
1,2,3	-	-	-	-	-	-	Illinois	4000/30d
1,2,3	-	-	-	-	CEO	MR/15d	Illinois	4000/30d
1,2,3	-	-	648A/686	Contact/1d	CEO	MR/15d	Illinois	4000/30d
1	CVI988	2000/1d	-	-	CEO	MR/15d	Illinois	4000/30d
1	Md5-BACΔMEQ	2000/1d	-	-	CEO	MR/15d	Illinois	4000/30d
2	HVT	2000/1d	648A	Contact/1d	CEO	MR/15d	Illinois	4000/30d
2	HVT+SB-1	1000+1000/1d	648A	Contact/1d	CEO	MR/15d	Illinois	4000/30d
2	CVI988	2000/1d	648A	Contact/1d	CEO	MR/15d	Illinois	4000/30d
3	HVT/CVI988	In ovo/1d	686	Contact/1d	CEO	MR/15d	Illinois	4000/30d
3	HVT + CVI988	In ovo	686	Contact/1d	CEO	MR/15d	Illinois	4000/30d
3	Md5-BACΔMEQ	1d	686	Contact/1d	CEO	MR/15d	Illinois	4000/30d

[a] Three experiments were conducted, all experiments included four control groups (above thick border line):1) A group that did not receive any treatment (negative control); 2) a group that only received ILT (will develop ILT); 3) a group that was vaccinated with CEO and challenged with ILT (will be protected against ILT); and a group that was challenged with MDV (648A or 686 strains) at day of age, vaccinated with CEO and challenged with ILTV (this group will show ILT even though it is vaccinated with CEO due to MDV-IS).

[b] Different MD vaccines were evaluated (HVT, HVT+SB1, CVI988, Md5-BACΔMEQ) administered either at 1-day-old or 18 days embryonation (in ovo). The dose in plaque forming unit (PFU) and the age at inoculation is indicated.

[c] vv+ MDV strains, 648A in experiments 1 and 2 and 686 in experiment 3, were used as MDV challenge. Infection occur at day of age by contact.

[d] CEO vaccine was used in all experiment at manufacturer's recommendation (MR) at 15 days old via drinking water.

[e] ILTV challenge was done with virulent strain Illinois strain (4000 PFU) at 30 days old via intratracheal inoculation.

Table 3.2. Oligonucleotide primer sets used for real time PCR and real time RT-PCR

Target gene	Sequence	Orientation
GAPDH	5'-GGAGTCAACGGATTTGGCC-3'	Forward
	5'-TTTGCCAGAGAGGACGGC-3'	Reverse
gB	5'-CGGTGGCTTTTCTAGGTTCG-3'	Forward
	5'-CCAGTGGGTTCAACCGTGA-3'	Reverse
Meq	5’-GGTCTGGTGGTTTCCAGGTGA-3’	Forward
	5’-GCATAGACGATGTGCTGCTGA-3’	Reverse
ICP4	5'-CGCCACACGAGAACACAATG-3'	Forward
	5'-GGTTGGAGTAGAGCTGCAACTGT-3'	Reverse
pp38	5'-GTGATGGGAAGGCGATAGAA-3'	Forward
	5'-AGCTACCCCTTTCGGTTTGT-3'	Reverse
28s	5′-GGCGAAGCCAGAGGAAACT-3′	Forward
	5′-GACGACCGATTTGCACGTC-3′	Reverse
mdv1-miR-M4-5p	5’- TTAATGCTGTATCGGAACCCTTC-3’	Forward
snoU83B	5’- GTTCGGTGATGAAACCATGGA -3’	Forward

Table 3.3. Protection of MD vaccines against MDV-induced tumors and correlation of MDV-induced tumor and pMDV-IS.

Exp	Treatment	PI CEO[a]	PI MD[b]	PI MDV DNA [b]	Correlation [c]		
					PI MD vs PI MDV DNA	PI MD vs PI CEO	PI MDV DNA load vs PI CEO
2	None/648A/CEO/ILTV	32.0	-	NA	NA	NA	NA
2	HVT/648A/CEO/ILTV	76.5	52	NA			
2	HVT+SB1/648A/CEO/ILTV	42.0	92	NA			
2	CVI988-A/648A/CEO/ILTV	35.4	74	NA			
3	None/686/CEO/ILTV	45.6	-	0	r=0.99 ($p<0.05$)	r=-0.4 ($p>0.05$)	r=0.5 ($p>0.05$)
3	HVT (IO)-CVI988-B(D1)/686/CEO/ILTV	53.9	100	91.3			
3	HVT+CVI988-B(IO)/686/CEO/ILTV	54.8	100	95.5			
3	Md5-BACΔMEQ/686/CEO/ILTV	95.2	100	98.9			

[a] Protective index of the CEO vaccine (PI CEO) measured the ability of vaccine CEO to protect against challenge with ILTV. Values are expressed relative to the PI CEO value of the control group -/CEO/ILTV in that particular experiment. Values can vary from 0 (CEO did not protect at all in that group) to 100 (CEO protected in this group as well as in the control group -/CEO/ILTV).

[b] Protection index of MD vaccines was evaluated by two methods: ability of a MD vaccine protocol to control against the development of gross tumors (PI MD) and ability of a vaccine protocol to protect against high load of MDV DNA in the feather pulp at the termination of the study (PI MDV DNA).

[c] Correlation between PI MD and PI MDV DNA, PI MD and PI CEO (MDV-IS), and PI MDV DNA and PI CEO ILT (MDV-IS) were calculated. Statistical significance was considered when $P<0.05$.

Table 3.4. Expression of various viral transcripts in chickens vaccinated with different vaccine protocols (Experiment 3).

Treatment	% chickens positive for MDV DNA in spleen[a]		MDV transcript (fold change relative to control) [b]				
	gB	Meq	ICP4	pp38	gB	Meq	mdv1-miR-M4-5p
None/686/CEO/ILTV	100	100	3.59E+07^{A}	1.94E+07^{A}	3.79E+06^{A}	2.10E+09^{A}	1.36E+15^{A}
HVT (IO)-CVI988 B (D1)/686/CEO/ILTV	100	100	9.59E+03^{B}	6.32E+04^{B}	4.05E+02^{B}	3.18E+06^{B}	3.53E+01^{B}
HVT+CVI988-B (IO)/686/CEO/ILTV	100	100	4.05E+03^{B}	2.56E+05^{AB}	3.47E+02^{B}	1.86E+06^{B}	2.80E+01^{B}
Md5-BACΔMEQ (D1)/686/CEO/ILTV	100	100	1.10E+04^{B}	9.44E+04^{AB}	1.25E+01^{B}	1.00E+00^{C}	1.00E+00^{B}

[a] Percentage of chickens (6 per treatment group) that had detectable levels of MDV genes gB and Meq by real time PCR at the termination of the study.

[b] MDV transcripts in the spleens (6 chickens per treatment group). Results are presented as fold difference relative to control group (None/None/None/None).

Comparison of different expression of MDV transcripts between each group are indicated by superscript capital letters; the same letter indicates that no statistically significant differences were found ($p<0.05$).

CHAPTER 4

Evaluation of factors that affect the development of late-Marek's disease virus-induced immunosuppression: virus pathotype and host sex

4.1 Abstract

Marek's disease virus (MDV) is a herpesvirus that induces lymphoma and immunosuppression in chickens. MDV induced immunosuppression (MDV-IS) is complex and can be divided into two phases, early-DV-IS associated with cytolytic infection in the lymphoid organs and late-MDV-IS that appears during the establishment of latency and tumors. We have recently developed a model to reproduce late MDV-IS under laboratory condition. This model evaluates late-MDV-IS indirectly by assessing the effect of MDV infection on the efficacy of infectious laryngotracheitis vaccines against challenge with infectious laryngotracheitis virus (ILTV), hence the name late-MDV-IS ILT model. In the present study we have used the late-MDV-IS ILT model to investigate the role of MDV pathotype and the sex of the host on the development of late-MDV-IS. We have evaluated 5 strains of MDV representing three different pathotypes: virulent (617A, GA), very virulent (Md5) and very virulent plus (648A, 686). Our results showed that only very virulent plus (vv+) strains were able to induce late-MDV-IS in this model. An immunosuppressive rank (IS rank) was established based on the ability of MDV to reduce efficacy of CEO vaccine (values go from 0 to 100, being 100 the highest immunosuppressive ability). The IS rank of the evaluated MDV strains ranged from 5.97 (GA) to 20.8 (617A) in the vMDV strains, 5.97 to 16.24 in the vvMDV strain Md5, and 39.08 to 68.2 in the vv+ strain 648A and 686. In this study both male and female chickens were equally susceptible to MDV-IS induced by vv+MDV 648A. Our findings suggest that increased immunosuppression ability is a feature that vv+ strain have acquired and might be related to increased virulence.

4.2 Introduction

Marek's disease (MD) is a lymphoproliferative disease of chickens that causes severe economic losses to the poultry industry. MD is induced by *Gallid herpesvirus 2* (1) (GaHV-2) or commonly known as Marek's disease virus (MDV). Three serotypes of MDV can be distinguished based on the serologic and genomic features of virus strains: Serotype 1 (GaHV-2) or MDV-1, non-oncogenic serotype 2 (GaHV-3) or MDV-2, and non-oncogenic serotype 3 (MeHV-1) or MDV-3 also known as herpesvirus of turkeys (HVT).

MD has increased in severity since it was first described in 1907 (2, 3). Originally, MD was described as a polyneuritis that appeared sporadically in old chickens causing inflammation of the peripheral nerves and negligible mortality (2, 4, 5). During the late 1950s, a more virulent form of MD was described and named as "acute MD" (6, 7). It was characterized by high mortality in young chickens and the development of lymphoma in peripheral nerves, skin, and various visceral organs (8, 9). The economic impact of "acute MD" was tremendous until the first vaccines were introduced (10-12). In the USA, vaccination against MD was first introduced in 1970s based on the FC126 strain of MDV-3 serotype (HVT) and successfully reduced morbidity and mortality caused by MDV (11). Within 10 year of HVT introduction, vaccine breaks were reported and virus isolated from these outbreaks were shown to have increased in virulence (13, 14). As MDV strains of greater virulence continued to be isolated, bivalent vaccine (combination of serotype 2 SB-1 and serotype 3 HVT) and serotype 1 CVI988 strain were introduced (14-18).

The ability of newly emergent MDV to break vaccine immunity was used by Witter to evaluate the virulence of various strains isolated from 1968 till 1997 (19). This study confirmed that MDV indeed had increased in virulence and further divided serotype 1 MDV into pathotypes: mildly virulent (mMDV), virulent (vMDV), very virulent (vvMDV) and very virulent plus (vv+MDV) (19).

Besides being able to break vaccine immunity, highly virulent MDVs have additional unique biological features. Gimeno et al demonstrated that vv and vv+MDV were more neurovirulent than vMDV and proposed a classification of MDV based on neurovirulence (20, 21). Classification in neuropathotypes could clearly differentiate between vMDVs and more virulent MDVs but failed to differentiate between vv and vv+MDVs (20). Highly virulent MDVs also tend to replicate faster and yield higher MDV DNA load in blood than lower virulent strains (22-24). Dunn et al proposed a classification of MDVs based on replication rate, however this criteria could clearly identify vMDV from more virulent strains but no differences were found between vv and vv+MDVs (24). Calnek et al suggested that virulence was related to an increase in their ability to induce lymphoid organ atrophy in chickens lacking maternal antibodies (MAb) (23). Viruses of greater virulence caused more severe atrophy of the Bursa of Fabricius and thymus than less virulent MDVs. A continuation of this study evaluating a large number of MDV strains of various pathotypes, however, failed to find a correlation between lymphoid organ atrophy and pathotype (25).

Several studies has been conducted to identify molecular markers that could be use to pathotype MDVs and that will aid in understanding the molecular basis of MDV increased

virulence. However, specific virus-encoded factors of MDV-1 that contributes to enhanced virulence are still unclear. Among all MDV genes, Marek's EcoRI-Q-encoded protein (Meq), major determinant of the oncogenicity of MDV-1, is the most extensively studied MDV-1 gene. Molecular changes of Meq have been shown to be associated with pathogenicity alteration of MDV-1, although none of these changes correlated with increase or decrease in virulence (26-32). Changes in other regions such as glycoprotein L (33, 34) and pp38 (26) has also been associated with increased in virulence but the exact correlation remains unknown. Recently Morgan et al. reported differential expression of MDV-1 microRNA in MDVs, with higher expression of mdv1-miR-M4 and mdv1-miR-M2 in highly virulent MDVs than in less virulent MDVs (35).

The mechanisms behind MDV evolution are uncertain. Possible explanations could be increased oncogenicity, increased immunosuppressive abilities, or a combination of both. Marek's disease induced immunosuppression (MDV-IS) is a complex phenomenon which is divided into early MDV-IS that occurs during early cytolytic infection in lymphoid organs and late MDV-IS that occur during the establishment of latency and tumors (23, 36-38). Early-MDV-IS is controlled by the presence of MAb and/or vaccination and it has much relevance in commercial flocks (39, 40). Late-MDV-IS, however, can occur in commercial chickens bearing MAb against MDV and even in chickens vaccinated against MD (38, 41). Recently, we have developed a model to study late-MDV-IS under laboratory conditions. This model indirectly evaluates late-MDV-IS by assessing the effect of MDV infection on the efficacy of infectious laryngotracheitis (ILT) vaccine on commercial meat type chickens (38). The objective of this study is to use the late-MDV-IS model to evaluate the effect of

MDV pathotype on the ability to induce late-MDV-IS. In addition, we also wanted to evaluate if the sex of the host has an effect on the development of late-MDV-IS. In the past, female chickens have been shown to be more susceptible to MD than males (42). Furthermore, in a previous experiment using the same genetic line, we have shown that females were more susceptible to MD after infection with vv+MDV strain 648A than males (43)

4.3 Materials and Methods

Chickens. Commercial specific pathogen free SPAFAS chickens (Charles River SPAFAS, N Franklin, CT) were used as MDV shedder chickens. Female commercial meat type chickens (grandparents) bearing MAb against MDV were used as experimental chickens in experiment 1 to 3. Male and female commercial meat type chickens bearing MAb against MDV were used as experimental chickens in experiment 4. Experimental chickens came from dams that were vaccinated with vaccines of the three serotypes (HVT, SB-1, and CVI988), therefore they were considered to have MAb against the three serotypes.

Viruses and vaccine. Commercial HVT (Merial Select, Inc., Gainesville, GA) was used to vaccinate shedder chickens at 18 day of embryonation following manufacturer's recommendations. MDV strains used as challenged were: 617A (v) (19), GA (v) (44), Md5 (vv) (45), 648A-ADOL and 648A-ATCC (vv+), (19), and 686 (vv+) (19). Information about the passage history of each of the virus strain used in each experiment is presented in Table 4.1. Chicken embryo origin (CEO) vaccine strain of ILTV was obtained from a commercial

manufacturer (Merial Select, Inc., Gainesville, GA, USA). Illinois-N71851 strain of ILTV that has been characterized as a virulent ILTV strain were used for LT challenge (46).

Real time RT-PCR. RNA was extracted from spleen samples using the Perfect Pure RNA tissue kit (5-Prime Inc, Gaithersburg, Maryland, USA) following manufacturer's recommendations. Transcription of MDV genes Meq and pp38 and chicken housekeeping 28S was evaluated by real time RT-PCR using specific primers as reported (47). Primer sequence is listed in Table 4.2. Amplifications were done in an Mx3005 Stratagene (Stratagene, La Jolla, California, USA) thermocycler by amplifying 25µl reactions using Brilliant II SYBR® Green Q-PCR Master Mix (Agilent Technologies, Santa Clara, CA, USA). The profile cycles used were 1 cycle of 50°C for 30 min; 1 cycle of 95°C for 10 min; 50 cycles of 95°C for 10 sec and 56°C for 1 min; and 1 cycle of 95°C for 1 min, 55°C for 30 sec, and 95°C for 30 sec. The melting curves was obtained at the end of amplification by cooling the sample at 2.0^{o}C/s to 60^{o}C and then increasing the temperature to 95^{o}C at 0.1^{o}C/s. Samples were ran in duplicates and the Ct was averaged. Average Ct value were subtracted from total number of cycles (50 - average Ct). Correction for differences in RNA levels between samples was done by using a 28S correction factor via the formula: (50 - overall mean Ct 28S rRNA-specific product from all samples) / (50 - average Ct for 28S rRNA-specific product for each sample). Corrected viral gene means was calculated using the formula: 28S correction factor X (50 Ct value for viral gene) X viral gene slope/28S gene slope) (48).

Analysis of microRNA expression by real-time PCR. First strand complementary DNA (cDNA) was synthesized from 1μg of polyadenylated total RNA from each spleen samples using a miRNA 1st strand cDNA synthesis kit (Agilent, Santa Clara, CA) following manufacturer's instruction. For miRNA real time PCR, each reaction contained 1 μl of cDNA and 24 μl High-Specificity miRNA QPCR Core Reagent Kit Master Mix (Agilent Technologies, Santa Clara, CA, USA). PCR was performed using the following conditions: 95°C for 15 min followed by 40 cycles of [95°C 10 sec and 60°C 20 sec] using Mx3005 Stratagene (Stratagene, La Jolla, California, USA) thermocycler. Expression of MDV miRNAs mdv1-miR-M4-5p, mdv1-miR-M2-3p, and chicken miRNA snoU83B was conducted using forward primers previously reported (49). Primer sequence is listed in Table 4.2. Average Ct value were subtracted from total number of cycles (40 - average Ct). Correction for differences in miRNA levels between samples was done by using a snoU83B correction factor via the formula: (40 - overall mean Ct snoU83B miRNA-specific product from all samples) / (40 - average Ct for snoU83B miRNA product for each sample). Corrected viral gene means was calculated using the formula: snoU83B correction factor X (40 Ct value for viral gene) X viral gene slope/ snoU83B gene slope).

Clinical Signs. Each bird was identified and observed daily for clinical signs of ILT such as gasping, coughing, sneezing, conjunctivitis and expectoration. Mortality was also recorded.

Development of ILT clinical sings (CS) was assessed by ILT-CS score as previously described (38). Briefly, ILT-CS score was calculated as the total number of days a particular chicken showed clinical signs (range from 0 to 6).

Gross Lesions. Upon necropsy, dead birds were evaluated for ILT and MD lesions. ILT gross lesions (GL) were scored from 0 to 4 in a gradient of severity. The criteria was as follow: 0 = normal, 1 = presence of light mucus, 2 = congested, thick mucus or bloody mucus, 3 = presence of caseous exudate, and 4 = tracheal plugs.

MD lesions were observed by evaluating presence of tumors in nerves and viscera as well as MD lesions in eye and skin.

Assessment of late-MDV-IS. Evaluation of late-MDV-IS was done based on ILT clinical signs (CS) and gross lesions (GL) as previously described (38). Number of days exhibiting CS was calculated for each chicken (values 0-6). GL in trachea at termination were scored based on severity (values 0-4). Three indexes were calculated to assess the severity of late-MDV-IS: ILT index, protection index, and immunosuppression rank

ILT index was calculated to assess severity of the disease based on CS and GL.

ILT index (ILTI) = CS + GL (values 0-10)

Protection index of the CEO vaccine (PI CEO) was calculated using ILTI values from the positive control group (-/ILTV) and the CEO vaccinated groups (-/CEO/ILTV, and MDV/CEO/ILTV) to measure protection provided by CEO vaccine.

$$PI\ CEO = (ILTI_{-/ILTV} - ILTI_{TXT}) / ILTI_{-/ILTV}) \times 100$$

$ILTI_{-/ILTV}$ is ILT index of -/-/ILTV control group.

$ILTI_{TXT}$ is ILT index of any treatment group that had been vaccinated with CEO vaccine.

Immunosuppression rank was calculated using PI CEO values of groups -/CEO/LTV and MDV/CEO/LTV to evaluate the effect of late-MDV-IS on protection induced by CEO vaccination.

$$\textit{Immunosuppression rank (IS rank)} = 100 - ((PI_{MDV/CEO/ILTV} / PI_{CEO/ILTV}) \times 100)$$

Experimental Design. Animal experiments were conducted following the guidance and under approval of North Carolina State University Institutional Animal Care & Use Committee (IACUC). Four experiments were conducted using the late-MDV-IS ILT model as described (38). Briefly, SPAFAS chickens were vaccinated with HVT vaccine *in ovo* via amniotic route at 18 days of embryonation (ED) to avoid the development of transient paralysis and to ensure survival. At hatch, shedder chickens were inoculated subcutaneously with 500 PFU of one of the MDV strains evaluated and housed in isolation for 15 days prior to the beginning of experiment. All chickens infected with different MDV strains were placed in separate isolation rooms.

In each experiment, newly hatched experimental meat type chickens were wing banded, divided into groups and placed in environmentally controlled rooms (BSL-2) for the rest of the experiment. Each treatment group consisted of 40-42 experimental chickens. In experiment 4, each treatment group were done in replicates one including males and the other including females. Chickens were divided into several different groups (Table 4.1). Each experiment included three control groups, 1) Group 1 received no treatment; 2) Group 2 was challenged with ILTV; and 3) Group 3 was vaccinated with CEO and challenged with ILTV. Chickens of other groups were infected with MDV strains 617A (v), GA (v), Md5 (vv),

648A-ADOL (vv+), 648A-ATCC (vv+) or 686 (vv+) by contact exposure, were vaccinated with CEO and challenged with ILTV. Due to logistics, it was not possible to evaluate all MDV strains in one single experiment. Therefore, for each experiment results are presented relative to the controls of that particular experiment. In addition, three strains (Md5, 648A and 686) were evaluated in more than one experiment. Details of each experiment are included in Table 4.1.

At 15 days of age, experimental meat type chickens were vaccinated with CEO vaccine through drinking water per manufacturer's recommendations. Two weeks later (day 30) meat type chickens were identified with color leg bands and challenged intratracheally with 2000 PFU of ILTV. Every chicken was monitored daily for ILTV clinical signs during six days starting after challenge with ILTV. At day 7 following ILTV challenge (termination), all chickens were euthanized. Chickens were necropsied at termination or at time of death and gross lesion of ILT and MD were examined. At termination of Experiment 2, 3 and 4, spleen of six chickens per group were sampled for nucleic acid extraction.

Data analysis and statistic. Data were analyzed with statistical program Statistica (Stat Soft, Tulsa, Oklahoma, USA) and SPSS (IBM, New York, USA). Comparison between two groups were conducted using Student's t test while comparison between more than two groups were conducted using one-way analysis of variance (ANOVA) test. The Scheffe test was used as a post hoc analysis. The level of significance was $p<0.05$.

4.4 Results

Effect of pathotype on late-MDV-IS. Five MDV strains representing three different pathotypes (v, vv, and vv+) were evaluated. The negative effect of each MDV strain on the efficacy of CEO is presented (Figure 4.1). Results are presented relative to the PI CEO vaccine in the control group (None/CEO/LTV) of their respective experiment. All groups inoculated with vv+ strains of MDV (648A-ATCC, 648A-ADOL, 686-1 and 686-2) had a significant reduction in the protection index of CEO when compared to the control group None/CEO/LTV of their respective experiments. No statistical significant differences were found in the PI CEO between other strains of MDV (v and vv) and the control group (None/CEO/LTV) of their respective experiments.

IS rank values were calculated as described in Materials and Methods and results are presented in Figure 4.1. vv+ MDV strains had higher IS rank values ($648A_{ADOL}$= 68.2; 648_{AATCC} = 38.7; 686 = 67.8 and 54.4) than v (617A = 20.4 and GA = 13) and vv (Md5 = 6 and 15.6) MDV strains.

Effect of pathotype on the development of tumors. MD lesions were evaluated during necropsy in all experiments (Figure 4.2). Frequency of gross tumors observed at 35 days varied between strains but also between replicates of the same strain. Frequency of tumors tend to be higher in groups inoculated with vv+MDV than in other groups; the largest frequency of tumors was found in chickens inoculated with 686 (experiment 3) and with 648A-ATCC (86% in both cases), followed by strain 686 used in experiment 4 (75%). Chickens inoculated with 648A-ADOL had lower frequency of chickens with tumors (50%)

than strain 648A-ATCC (86%). Groups inoculated with vvMDV Md5 had variable frequency of tumors ranging from 10% in experiment 1 to 58% in experiment 2. Strain 617A (617A) induced tumors in 48% of the chickens and vMDV GA induces the lowest percentage of tumor (11%).

Effect of MDV pathotype on the transcription of various MDV genes and expression of viral microRNAs. Transcription of various MDV genes and transcription of two MDV miRNA (mdv1-miR-M4-5p and mdv1-miR-M2-3p) were evaluated in experiments 2, 3 and 4. Results are presented in Figure 4.3. Most groups had similar levels of all evaluated transcripts and microRNA except groups infected with GA and with 686. Groups inoculated with GA had the lowest levels of pp38, and meq transcripts and there was no expression of mdv1-miR-M4-5p and mdv1-miR-M2-3p. By contrast, strain 686 had the highest levels of meq transcripts and the highest expression of mdv1-miR-M4-5p and mdv1-miR-M2-3p. Such differences were more remarkable in experiment 4 than in experiment 3.

Comparison of host sex susceptibility to late-MDV-IS. Effect of host sex on the ability of MDV to induce late-MDV-IS was evaluated in Experiment 4. Equal numbers of male and female experimental chickens (40) were infected with 686 (vv+) strain of MDV (Figure 4.4). Data presented are relative to the protection conferred by the CEO vaccine on the control group None/CEO/LTV. No significant differences ($P<0.05$) were found between male (60%) and female (52%) chickens on their susceptibility to late-MDV-IS.

4.5 Discussion

MDV is a potent immunosuppressor in chickens. In previous work, we have demonstrated that late-MDV-IS can occur in vaccinated chickens that are properly protected against MDV-induced tumors and in absence of lymphoid organ atrophy (38, 41). Late-MDV-IS affect cell mediated immune responses (38) as well has humoral immune responses (41). In the present work, we have shown that MDV pathotype but no host sex affects the ability of MDV to induce late-MDV-IS. Only the two vv+MDV strains used in this study (648 and 686) were able to induce late-MDV-IS. These results suggest that increased immunosuppressive ability is a unique feature of vv+MDV that might be involved in the increased virulence of MDV.

Several attempts have been done to relate MDV-IS and virulence, however the relationship between MDV-IS and MDV virulence remains elusive. Calnek et al found correlation between early-MDV-IS (lymphoid organ atrophy) and pathotype (23). However, extension of this work evaluating large number of MDV strains failed to correlate lymphoid organ atrophy to MDV virulence (25). Previous studies have focused on the early-MDV-IS that occurs in chickens lacking MAb against MDV. In the present study we have demonstrated that pathotype has an effect on late-MDV-IS and proposed that late-MDV-IS might be responsible, at least partly, of the evolution of MDV towards more virulence. It was particularly interesting that strain 648A obtained directly from ADOL had higher IS rank than strain 648A obtained from ATCC. There are several explanations for such findings. One possibility could be the passage history between both strains. 648A-ADOL had 12 passages

in chicken embryo fibroblast and 648A-ATCC had 10 passages in duck embryo fibroblast followed by an unknown number of passages in the chicken fibroblast line SL-29 and one passage in chicken kidney cells. It is possible that differences in the history passage might have contributed to differences in the ability to induce MDV-IS but not in the ability to induce tumors. Previous studies have shown that 648A strain loses the ability to induce transient paralysis between passages 30 and 40 in DEF and the ability to induce tumors between passages 80 and 100 in DEF (50). Another explanation for such differences could be variability among replicates. Variation between replicates is often seen in MDV experiments (19, 25). Variation in virulence rank data for prototype strains JM/102W (8.0 to 32.2) and Md5 (39.5 and 69.0) was seen even under identical experimental condition (25). This was possibly the case for strain Md5 in our experiment since the same strain with identical passage history was used in experiments 1 (IS rank 5.97) and 2 (16.24). In any case, regardless of the cause for the differences within the same virus strain, differences in the PI CEO between that strain and the control group (-/CEO/LTV) and therefore, it can be safely concluded that in this study only vv+MDV but not strain of other pathotypes induced late-MDV-IS.

The effect of pathotype in the development of tumors in the present study is not clear since great variability was found not only between strains but also between experiments. However, results should be analyzed with caution since the experiment was terminated at 35 days and evaluation of tumors was conducted only by gross inspection. It is likely that some chickens that were developing tumors still did not show macroscopic lesions that could be detected at necropsy. Nonetheless it is remarkable that groups that already had high levels of

tumors (617A = 48%, Md5 in the second experiment = 58%) failed to induced late-MDV-IS. In previous studies we have demonstrated that controlling MDV-induced tumors is not enough to protect against late-MDV-IS (41). The results of this study suggest that development of tumors is not related to late-MDV-IS, either. Further studies to elucidate the mechanisms behind late-MDV-IS are warranted.

Pathotype did not have a major effect on the transcription of MDV genes meq and pp38. Differences were only observed in the two extremes of the virulence spectrum (GA and 686). However, differences between vv+ strains and less virulent viruses were detected in the expression of microRNAs (mdv1-miR-M4-5p and mdv1-miR-M2-3p). Our results are in agreement with previously studies conducted by Morgan et al. (35). They reported that highly virulent MDVs had higher level of mdv1-miR-M4 and mdv1-miR-M2 than less virulent strains. miRNAs are short (~19-24 nucleotides) RNAs that are transcribed from noncoding genomic regions and function as post-transcriptional regulators (51). mdv1-miR-M4 is a well characterized miRNA of MDV-1 that shares the same seed sequence with chicken gga-miR-155 and also with Kaposi's sarcoma herpesvirus microRNA kshv-miR-K12-11 (35, 52, 53). Mdv-mir-M4-5p was identified to be highly expressed in tumors (35) and it is involved but not essential in MDV induced tumorigenesis (54, 55). mdv1-miR-M2-3p is another miRNA in the Meq miRNA cluster located next to mdv1-miR-M4-5p. Previous study by Goher et al. (56) identified mdv1-miR-M2-3p binding site to be cluster of differentiation 86 (CD86) and cluster of differentiation 80 (CD80). They hypothesized that mdv1-miR-M2-3p might be involved in T cell activation and responsible to the tumor resistance on chickens vaccinated with HVT (56). In our study, expression of both mdv1-

miR-M4 and mdv1-miR-M2-3p was higher in groups inoculated with vv+MDV strains than in groups inoculated with less virulent MDVs. The relevance of these microRNAs on the development of late-MDV-IS warrant further studies.

The gold standard method to pathotype MDV isolates is the biological assay proposed by Witter (19), commonly known as Avian Disease and Oncology Laboratory (ADOL) pathotyping assay. It is based on the ability of MDV to break immunity conferred by two commercial vaccines, HVT and HVT+SB1, in comparison to prototype MDV strains (19). Because pathotyping assay are costly and labor-intensive several attempts have been done to simplify the process. Neuropathotyping was described as an alternative to characterize MDV virulence and could differentiate clearly between vMDV from vv/vv+MDV (20). However, differences between vv and vv+ could not be detected for many isolates (20). A similar situation occurred when using other criteria such as lymphoid organ atrophy, virus replication, or even molecular features (24, 25, 32). Our study show one feature, ability to induce late-MDV-IS, which seems to be unique to vv+MDV. Further studies are warranted to understand the mechanism by which vv+MDV but not v or vv MDVs are able to induce late-MDV-IS. A better understanding of this pathogenic feature might allow us to find an easier alternative to pathotyping and to better understand MDV evolution.

Our study show that males and females are equally susceptible to late-MDV-IS induced by vv+MDV. Previous studies have demonstrated that female chickens are more susceptible to the development of tumors than males (42). Furthermore, females of the same genetic line as the one used in this study have been shown to be more susceptible to MD after

infection with vv+MDV strain 648A than males (43). In the present study, we could not demonstrate any difference in susceptibility between males and females to the development of late-MDV-IS. These results suggest once more that MDV-induced tumors and late-MDV-IS follow different mechanisms. However, further studies need to be conducted to confirm that this is the case.

In summary, this study demonstrated that only vv+MDV are capable to induce late-MDV-IS in commercial chickens using the late-MDV-IS ILT model. Viruses of other pathotypes that induced tumors at various frequencies did not induce late-MDV-IS. No differences in the transcripts of meq or pp38 could be detected between strains that induces late-MDV-IS (vv+MDV) and those that did not (v and vv). However, expression of two microRNAs (mdv1-miR-M4-5p and mdv1-miR-M2-3p) was higher in groups inoculated with vv+MDV. Further studies are warranted to evaluate the role of those microRNAs on the development of late-MDV-IS and if they could be used to identify vv+MDV isolates.

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4.7 Figures and tables

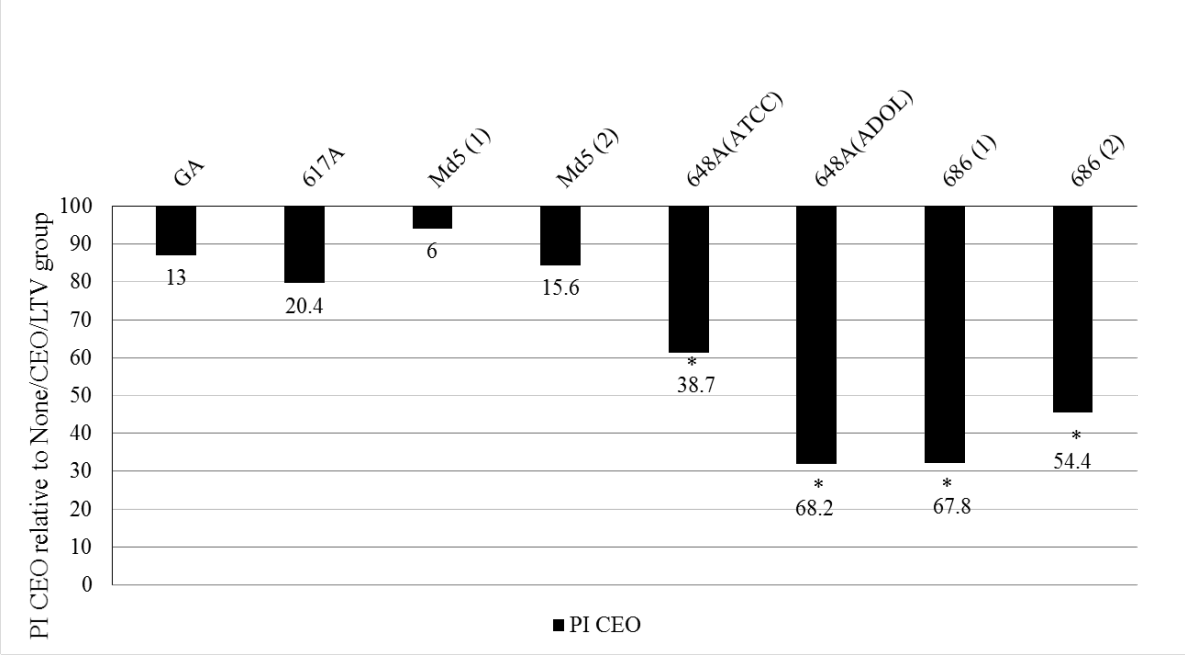

Figure 4.1. Effect of various MDV strains on the efficacy of CEO vaccine. Results are presented as the protective index of CEO (PI CEO) for each treatment group relative to the values of the PI CEO in the control group None/CEO/LTV for that particular experiment. Strains that are followed by a number in brackets indicate that they were included in more than one experiment as indicated in Materials and Methods. Asterisk (*) under the bars indicate that the PI CEO value in that group was significantly different ($p<0.05$) to the PI CEO in the control group None/CEO/LTV of the same experiment. Number below the bars indicate the immunosuppressive rank calculated as:

IS rank = 100 – ((PI CEO $_{MDV/CEO/ILTV}$ / PI CEO $_{-/CEO/ILTV}$) X 100).

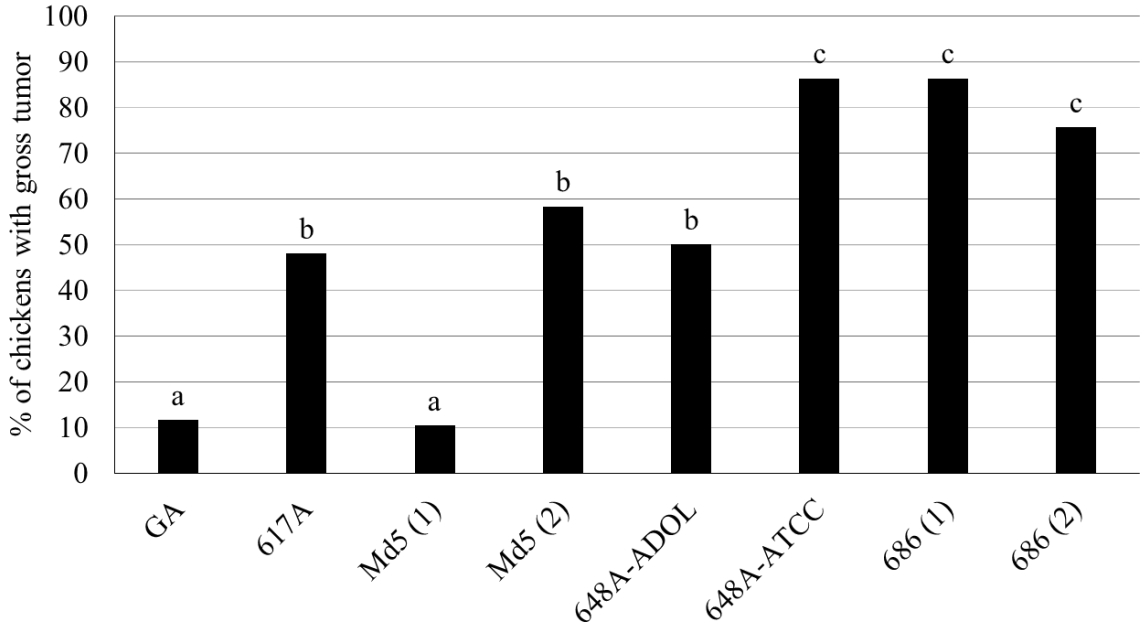

Figure 4.2. Frequency of chickens that develop MDV-induced tumors by the end of the experiment (35 days) following inoculation with various MDV strains. Values identified by different letters are statistically significant between treatment ($p<0.05$).

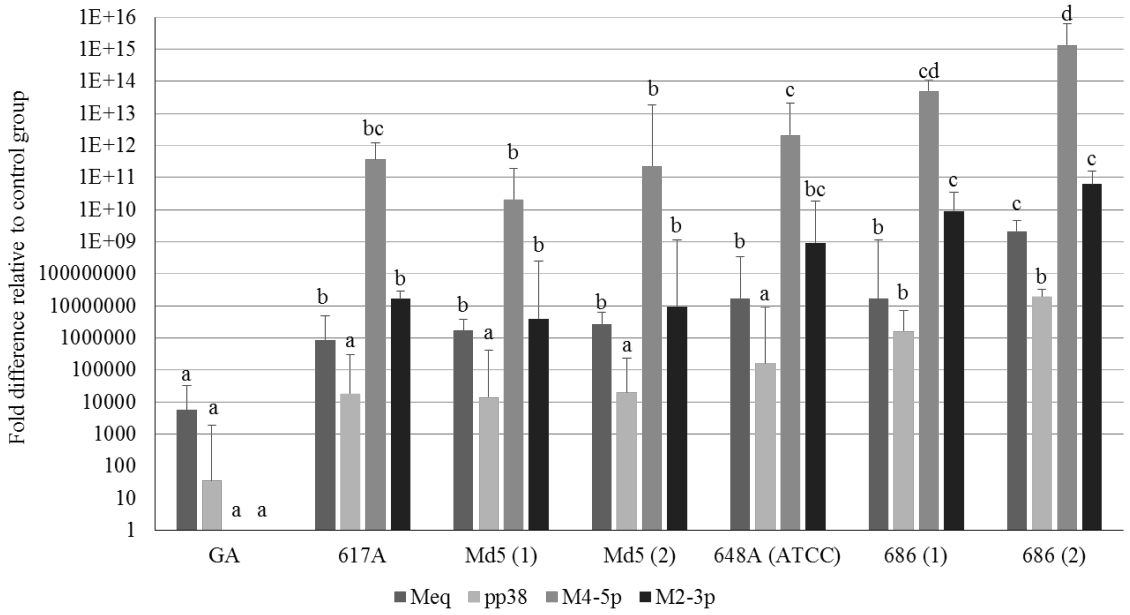

Figure 4.3. Evaluation of MDV transcripts (Meq and pp38) and expression of MDV miRNAs (miR-M4-5p and miR-M2-3p) in the spleen of chickens (six per treatment group) infected with various MDV strains by contact at day of age. Data was normalized to internal controls 28S and snoU83B. Results are presented as fold difference relative to control group (None/None/None/None). Different alphabet on top of bar indicate that differences were statistically significant ($p<0.05$).

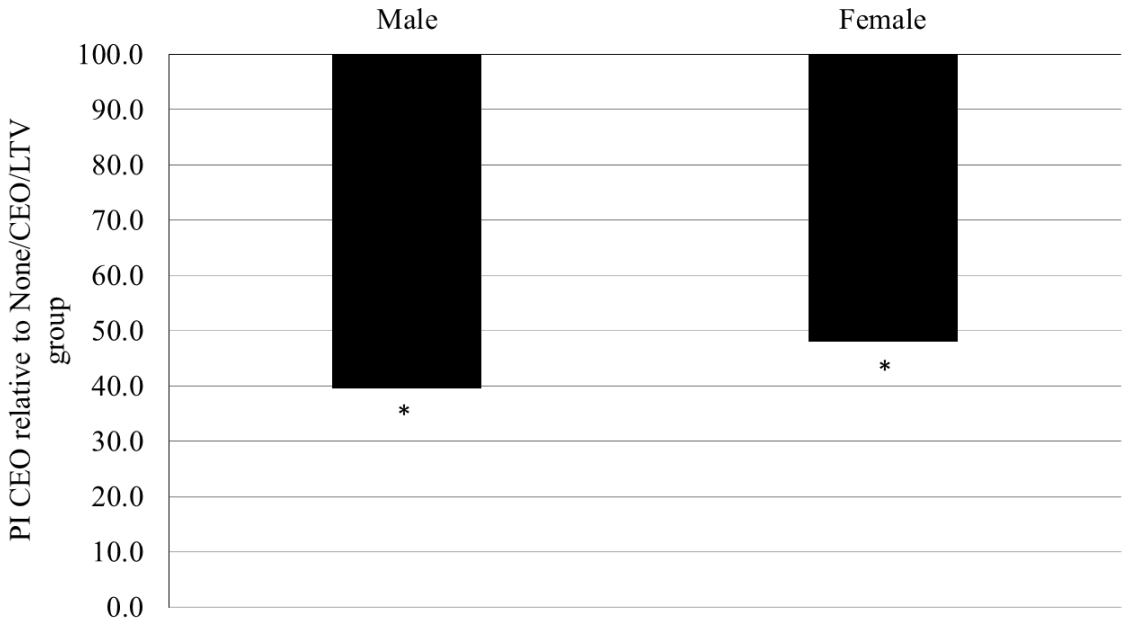

Figure 4.4. Effect of host sex on the susceptibility to late-MDV-IS (Experiment 4). Results are presented as the protective index of CEO (PI CEO) for each treatment group relative to the values of the PI CEO in the control group None/CEO/LTV. Asterisk (*) under the bars indicate that the PI CEO value in that group was significantly different ($p<0.05$) to the PI CEO in the control group None/CEO/LTV.

Table 4.1. Experimental design

Exp[1]	MD challenge			LTV vaccination and challenge			
	MDV isolate[2]	Passage history	Dose/Age[2]	Vaccine[3]	Dose/Age[3]	Challenge[4]	Dose/Age[4]
1-4	-	-	-	-	-	-	-
1-4	-	-	-	-	-	Illinois	4000/30d
1-4	-	-	-	CEO	MR/15d	Illinois	4000/30d
2	GA (v)	21 (DEF)	Contact/1d	CEO	MR/15d	Illinois	4000/30d
3	617A (v)	8 (DEF)	Contact/1d	CEO	MR/15d	Illinois	4000/30d
1	Md5 (1) (vv)	7 (DEF) + 2 (CKC)	Contact/1d	CEO	MR/15d	Illinois	4000/30d
2	Md5 (2) (vv)	7 (DEF) + 2 (CKC)	Contact/1d	CEO	MR/15d	Illinois	4000/30d
1	648A-ADOL (vv+)	12 (CEF)	Contact/1d	CEO	MR/15d	Illinois	4000/30d
3	648A-ATCC (vv+)	10 (DEF) + unknown (SL-29) + 1 (CKC)	Contact/1d	CEO	MR/15d	Illinois	4000/30d
3	686 (1) (vv+)	10 (DEF) + 1 (CKC)	Contact/1d	CEO	MR/15d	Illinois	4000/30d
4	686 (2) (vv+)	10 (DEF) + 2 (CKC)	Contact/1d	CEO	MR/15d	Illinois	4000/30d

[1] Four experiments were conducted, all experiment included three control groups (above thick border line): negative control, LTV control and CEO vaccine control.

[2] MDV strains representing three pathotypes (v =virulent, vv= very virulent, vv+ = very virulent plus) were evaluated. Passage history (number of passages and type of cells) is indicated. DEF = duck embryo fibroblast; CEF = chicken embryo fibroblast; CKC = chicken kidney cells; SL-29 is a chicken fibroblast line (ATCC® CRL-1590™)

[3] CEO vaccine were used in all experiments at manufacturer's recommendation (MR) at 15 days old via drinking water.

[4] LTV challenge were conducted using virulent strain Illinois strain (4000pfu) at 30 days old via intratracheal inoculation.

Table 4.2. Primers sequence

Target gene	Sequence	Orientation
Meq	5’-GGTCTGGTGGTTTCCAGGTGA-3’	Forward
	5’-GCATAGACGATGTGCTGCTGA-3’	Reverse
pp38	5'-GTGATGGGAAGGCGATAGAA-3'	Forward
	5'-AGCTACCCCTTTCGGTTTGT-3'	Reverse
28s	5′-GGCGAAGCCAGAGGAAACT-3′	Forward
	5′-GACGACCGATTTGCACGTC-3′	Reverse
mdv1-miR-M4-5p	5’- TTAATGCTGTATCGGAACCCTTC-3’	Forward
mdv1-miR-M2-3p	5’- CGGACUGCCGCAGAAUAGCUU-3’	Forward
snoU83B	5’- GTTCGGTGATGAAACCATGGA -3’	Forward

CHAPTER 5

Role of Tumors on the Development of Late-Marek's Disease-Virus-Induced Immunosuppression

5.1 Abstract

Marek's disease virus (MDV) induces a variety of syndromes in chickens that can be divided into neoplastic (lymphomas) and non-neoplastic (transient paralysis, lymphodegenerative disease, early mortality syndrome, and late paralysis). In addition, MDV induces severe immunosuppression (MDV-IS) that is complex and can be divided into two phases: early-MDV-IS which is associated with replication of MDV in the lymphoid organs and it is unrelated to tumors; and late-MDV-IS that occurs once latency gets establishes. The pathogenesis of late-MDV-IS is poorly understood and it is unclear if it is related to the development of tumors. The objective of this study was to evaluate if MDV-induced tumors play a role on the development of late-MDV-IS. Two approaches were taken in this study. The first approach was to evaluate if deletion of meq, MDV oncogene, results in reduction of late-MDV-IS by using a model previously described (ILT model). Two animal experiments were conducted. In experiment 1, very virulent strain MDV Md5, a molecular clone Md5-BAC, and a recombinant Md5-BAC without the two copies of meq (Md5-BACΔMeq) were used. In experiment 2 vv+MDV strain 686, a molecular clone 686-BAC, and a recombinant 686-BAC lacking both copies of meq 686-BACΔMeq were used. The second approach was to conduct a retrospective study using results from 4 animal experiments involving 16 different treatment groups and 487 chickens. Correlation between development of MD tumors and late-MDV-IS was evaluated. The role of meq on the development of late-MDV-IS could not be assessed because none of the Md5 derived viruses nor 686-BAC and 686-BACΔMeq induced late-MDV-IS. Differences in the immunosuppressive abilities between 686 and 686-BAC might be due to differences in virulence and deserve further studies. No

correlation between development of tumors and late-MDV-IS was detected in the retrospective study.

5.2 Introduction

Marek's disease virus (MDV) is an alpha-herpesvirus that induces a variety of syndromes in chickens (1, 2). MDV-induced syndromes are divided into two major categories, those related to the development of tumors (lymphoma in viscera, skin, and nerves) and those that are unrelated to tumors (i.e. transient paralysis and lymphodegenerative syndromes) (3-5). In addition, MDV is able to induce severe immunosuppression (MDV-IS) but the pathogenesis of the latter is poorly understood.

Development of tumors is commonly known as Marek's disease (MD), lead to important economic losses, and therefore it is the best studied MDV-induced syndrome. MDV-induced tumors can appear as early as 12-14 days after infection of genetically susceptible young chickens with a virulent strain of MDV (6, 7). Lymphoma could occur in various organs including visceral organs, muscle, peripheral nerves, eyes and skin (8). Several factors influences the development of tumors such as genetic resistance, age at infection, host sex, MAb status, vaccine immunity and virus pathotype (9-13). Concurrent infection with other immunosuppressive diseases (i.e. chicken infectious anemia) also increases the incidence of MD tumors (14, 15). The composition of MD lymphomas is complex and includes mainly T lymphocytes (transformed and not neoplastically transformed) and a variable amounts of B lymphocytes, natural killer cells, macrophages, and other non-lymphoid cells (16-18). The main cell target for MDV transformation is

CD4+CD8- T cells (19). However, in chickens depleted of CD4+ cells, MDV can transform other lymphoid populations such as CD4-CD8+ and even CD4-CD8- cells (19-21). MDV transformed lymphocytes have high expression of CD30 (22), high MDV DNA load (22, 23), high expression of MDV oncoprotein meq (23, 24), and high expression of MDV microRNA mdv1-miR-M4-5p (24, 25).

Meq is a MDV oncogene (26). Xie et al (27) has shown that Meq is required for maintenance of transformed status of MDV-transformed lymphoblastoid cell lines (MDCC-MSB1). Liu et al (28) provided evidence that over expression of Meq resulted in the transformation of rodent fibroblast cell line. The strongest evidence of meq being the principal oncogene was provided by Lupiani et al (29) that show how deletion of both copies of Meq gene resulted in full abrogation of the transforming ability of vvMDV strain rMd5. Oncoprotein meq includes an N-terminal bZIP domain and a prolein-rich C-terminal transactivation domain (30). It is similar to oncoproteins such as Fos and Jun and can dimerize with itself of with other leucine ZIP proteins (31).

MDV-IS can be divided into two phases, early-MDV-IS associated with early cytolytic infection of lymphoid organs and late-MDV-IS that occurs during the establishment of latency and tumor development (32). Early-MDV-IS can be controlled by MAb against MDV and vaccination and it is not considered to have any relevance in commercial poultry (33). The mechanisms behind late-MDV-IS, however, are poorly understood. Several studies have demonstrated that tumor cells might contribute to MDV-IS. Addition of MDV lymphoblastoid cells to normal spleen cells inhibited the proliferation response to mitogen

(34). It is also possible that expression of inappropriate antigens on the surface of tumor cells contribute to MDV-IS (32). Ohashi et al shows that chicken fetal antigen expressed in the surface to tumor cells resulted in reduced NK activity of specific-pathogen-free (SPF) chickens spleen cells in vitro (35). On the other hand, MDV is able to induce MDV-IS by other mechanisms unrelated to tumors such as dysregulation of cytokines production (36), downregulation of MHC I (37, 38), downregulation of CD8 antigen (39) and involvement of microRNA (40). Furthermore, in previous studies we have demonstrated that controlling the development of tumors by vaccination was not sufficient to control late-MDV-IS, suggesting that tumors might not be related to late-MDV-IS (41).

The objective of the present study was to evaluate the role of tumors on the development of late-MDV-IS. Two different approaches have been used: (1) to study the effect of meq on the development of late-MDV-IS; and (2) to conduct a retrospective study to correlate development of tumors and late-MDV-IS.

5.3 Materials and Methods

Chickens. Commercial specific pathogen free SPAFAS chickens (Charles River SPAFAS, N Franklin, CT) were used as MDV shedder chickens. Female commercial meat type chickens bearing MAb were used as experimental chickens.

Viruses and vaccine. Turkey herpesvirus (HVT) FC-126 strain (Merial Select, Inc., Gainesville, GA) were used to vaccinate shedder chickens at 18 day of embryonation. Serotype 1 MDV strain, vvMDV Md5 (4), Md5-BAC (42, 43), Md5-BACΔMEQ (42, 43), vv+MDV 686 (6), 686-BAC (44) and 686-BACΔMEQ (Reddy et al, unpublished) were used

as MDV challenge. Passage history in cell culture of each of the MDV strains used is shown in Table 5.1. Chicken embryo origin (CEO) vaccine strain of ILTV was obtained from a commercial manufacturer (Merial Select, Inc., Gainesville, GA, USA). Illinois-N71851 strain of ILTV that has been characterized as a virulent ILTV strain were used for ILT challenge (45).

Experimental Design. Animal experiments were conducted following the guidance and under approval of North Carolina State University Institutional Animal Care & Use Committee (IACUC). Two experiments were conducted using the ILT model experimental design as described (46). Briefly, shedders chickens were vaccinated *in ovo* with HVT vaccine via amniotic route at 18 days of embryonation (ED) to avoid the development of transient paralysis and to ensure survival. At hatch, shedder chickens were infected with 500 PFU of vvMDV Md5 strain (Experiment 1) or vv+MDV 686 (Experiment 2) strain subcutaneously and maintained in isolation for 15 days prior to the beginning of experiment. Experimental chickens housed with the shedders were tested by real time PCR for both MDV and HVT at 7 days and at the end of the experiment. Horizontal transmission of MDV was confirmed by detecting MDV DNA in the feather pulp of experimental chickens at 7 days of age. Horizontal transmission of HVT from the shedders to the experimental chickens did not occur in any of the experiments of this study.

In each experiment, newly hatched meat type experimental chickens were wing banded, divided into groups (42 chickens per group) and placed in environment controlled room (BSL-2) for the rest of the experiment. Details of the experiment are shown in Table

5.1. Each experiment included three control groups, 1) Group 1 received no treatment (negative controls); 2) Group 2 received only ILTV challenge; 3) Group 3 received CEO vaccination and ILTV challenge. In addition, there were several MDV challenged group as follows:

Experiment 1. Three experimental groups infected with either Md5, Md5-BAC, or Md5-BACΔMEQ at day of age, received CEO vaccination and ILTV challenge. Challenge with Md5 was done by contact using shedders as reported (46). However, challenge with molecular clones Md5-BAC and Md5-BACΔMEQ was done by subcutaneous injection at day of age to avoid the bias of potential shedding differences with Md5 strain.

Experiment 2. Three experimental groups infected with either 686, 686-BAC, or 686-BACΔMEQ at day of age, received CEO vaccination and ILTV challenge. Challenge with 686 was done by contact using shedders as reported (46). However, challenge with molecular clones 686-BAC and 686-BACΔMEQ was done by subcutaneous injection at day of age to avoid the bias of potential shedding differences with 686 strain.

When meat type chickens were 15 days of age, they were vaccinated with CEO vaccine via drinking water per manufacturer's recommendations. Two weeks later (day 30 of the experiment), chickens were challenged intratracheally with 2000 PFU of ILTV. At this time all chickens were identified with colored leg bands. Every chicken was monitored daily for six days between challenge with ILTV and the termination of the experiment to evaluate if clinical signs of ILT were present. All chickens were necropsied at termination (day 7 after

ILTV challenge) or at time of death, and evaluated for gross lesions consistent with ILT and MD.

Clinical Signs. Each bird was identified and observed daily for clinical signs of ILT such as gasping, coughing, sneezing, conjunctivitis and expectoration. Mortality was also recorded.

Development of ILT clinical sings (CS) was assessed by ILT-CS score as previously described (46). Briefly, ILT-CS score was calculated as the total number of days a particular chicken showed clinical signs (range from 0 to 6).

Gross Lesions. Upon necropsy, dead birds were evaluated for ILT and MD lesions. ILT gross lesions (GL) were scored from 0 to 4 as follow: 0 = normal, 1 = presence of light mucus, 2 = congested, thick mucus or bloody mucus, 3 = presence of caseous exudate, and 4 = tracheal plugs.

MD lesions were observed by evaluating for presence of tumors in nerves, visceral organs, eye and skin.

Assessment of late-MDV-IS. Evaluation of late-MDV-IS was done based on ILT clinical signs (CS) and gross lesions (GL) as previously described. Number of days exhibiting CS was calculated for each chicken (values 0-6). GL in trachea at termination were scored based on severity (values 0-4). ILT index was calculated to assess severity of the disease based on CS and GL.

ILT index (ILTI) = CS + GL (values 0-10)

Protection index of the CEO vaccine (PIO_{CEO}) was calculated using ILTI values from the positive control group (-/ILTV) and the CEO vaccinated groups (-/CEO/ILTV, and MDV/CEO/ILTV) to measure protection provided by CEO vaccine.

$$\textit{Protection index } (PI_{CEO}) = (ILTI_{-/ILTV} - ILTI_{TXT}) / ILTI_{-/ILTV}) \text{ X } 100$$

$ILTI_{-/ILTV}$ is ILT index of -/-/ILTV control group.

$ILTI_{TXT}$ is ILT index of any treatment group that had been vaccinated with CEO vaccine.

Results are presented as PI_{CEO} of the MDV challenged groups (MD vaccinated or unvaccinated) relative to the value of the PI_{CEO} of the control group (None/None/CEO/ILTV).

Retrospective study on correlation between tumors and late-MDV-IS. Data from four animal experiments evaluating late-MDV-IS by the ILT model were used in this study. All experiments used in this study have been reported elsewhere (41, 46, 47) and details are summarized in Table 5.3. There was a total of 16 different treatment groups and 487 chickens. Selected treatment groups were inoculated with MDV and could be either vaccinated against MD or unvaccinated. All groups receive CEO vaccination and ILTV challenge as indicated for experiments 1 and 2 of the present study. Each individual chicken was evaluated for the development of ILT (clinical signs and gross lesions) and for the development of MD tumors. PI CEO of each treatment group were calculated as previously

described (46). Correlation analysis was conducted to determine if MD gross tumor and late-MDV-IS (PI CEO) was related.

Data analysis and statistic. Data were analyzed with statistical program Statistica (Stat Soft, Tulsa, Oklahoma, USA) and SPSS (IBM, New York, USA). Comparison between two groups were conducted using Student's t test while comparison between more than two groups were conducted using one-way analysis of variance (ANOVA) test. The Scheffe test was used as a post hoc analysis. In retrospective study, correlation between tumor frequency and PI CEO was evaluated by Pearson's test using the SPSS (IBM, New York, USA) software. The level of significance was $P<0.05$.

5.4 Results

Role of meq on late-MDV-IS. None of the MDV strains derived from vvMDV Md5 (Md5, Md5-BAC, and Md5-BACΔMEQ) induced late-MDV-IS (Table 5.2). No significant differences were found between MDV challenged groups (Md5/CEO/ILTV, Md5-BAC/CEO/ILTV and Md5-BACΔMEQ/CEO/ILTV) and the control group None/CEO/ILTV (94%, 95% and 109% vs 100%). Similarly, neither strain 686-BAC nor 686-BACΔMEQ induced late-MDV-IS (Table 5.2). PI CEO of group 686/CEO/ILTV was significantly lower (46%) than that of the control group None/CEO/ILTV (100%). However, no significant differences were found between group 686-BAC/CEO/ILTV (84%) and 686-BACΔMEQ/CEO/ILTV (89%) with the control group None/CEO/ILTV (100%).

Role of meq on MDV-induced tumors. Meq deletion resulted in total abrogation of tumors in chickens inoculated with either Md5-BACΔMEQ or 686-BACΔMEQ (Table 5.2). No

differences in the frequency of tumors were found between Md5 and the molecular clone Md5-BAC. However, vv+MDV strain 686 induced higher frequency ($p<0.05$) of tumors (75.61%) than the molecular clone 686-BAC (48.83%).

Retrospective study on correlation between tumors and late-MDV-IS. Data of 4 experiments involving 487 chickens in 16 treatment groups were evaluated (Table 5.3). Gross tumor frequency in the different treatment groups ranged from 0% to 86.36%. PI CEO ranged from 31.7 to 90.66. No correlation was found between percentage of gross tumors and PI CEO (Pearson coefficient $r=-0.162$), $p>0.05$).

5.5 Discussion

MDV-IS can be divided into two phases. Early MDV-IS that occurs few days after MDV infection is attributed to the cytolysis of infected B cell and presence of suppressor macrophages (32). Late-MDV-IS occurs later, once the virus gets into latency, and the mechanisms are poorly elucidated. The objective of this study was to determine if tumors play a role on the development of late-MDV-IS. Our results show that presence of tumors does not always involve development of late-MDV-IS (686-BAC induced tumors in 48.83% of chickens but it did not induce late-MDV-IS). Furthermore no correlation was found between development of MD tumors and late MDV-IS in a retrospective study involving 487 chickens. Our results suggest that tumors might not be always related to the development of late-MDV-IS.

Meq is a viral oncogene (27-29). Deletion of meq (rMd5ΔMEQ and Md5-BACΔMEQ) resulted in total abrogation of the neoplastic ability of vvMDV rMd5 (based on

cosmids) (29) and the BAC clone Md5-BAC (48). In previous studies conducted by Reddy et al. (unpublished) and in the present study, deletion of meq (686-BACΔMEQ) also resulted in total abrogation of the neoplastic ability of vv+MDV strain 686-BAC (49). It was our objective to evaluate the role of meq on the development of late-MDV-IS by using two molecular clones of MDV (Md5-BAC and 686-BAC) and the respective recombinant strains with both copies of meq deleted (Md5-BACΔMEQ and 686-BACΔMEQ). Unfortunately none of the molecular clones induced late-MDV-IS and therefore it was not possible to evaluate the role of meq on late-MDV-IS. It is likely that Md5-BAC did not induce late-MDV-IS because of low virulence. In previous studies we have shown that vv+ but no vv or v MDVs were able to induce late-MDV IS (47). In the present study again none of the strains derived from Md5 induced late-MDV-IS. Surprisingly, 686-BAC did not induce late-MDV-IS although original strain 686 did. These results could be due to reduced virulence in the molecular clone 686-BAC when compared to the parental virus 686. In previous studies r686 induced lower frequency of tumors than 686 (44). In addition, 686-BAC has ranked lower virulence in the pathotyping assay than strain 686 (Dunn, personal communication). In this study, 686-BAC also induced significantly lower frequency of tumors (48.83%) than the parental virus 686 (75.61%). In previous studies, we have suggested that the ability of MDV strains to induce late-MDV-IS is associated with the most virulent isolates (47). Our results support this hypothesis and further studies using viruses at various levels of attenuation are warranted. Furthermore, comparison of strain 686 and 686-BAC could be very valid to better understand the mechanisms of late-MDV-IS and the evolution of MDV.

Correlation analysis between tumors and PI CEO in a retrospective study showed that tumors were not associated with development of late-MDV-IS. This result supports previous finding that suggest lack of correlation between MD tumors (measured by gross inspection and MDV DNA load) and late-MDV-IS (measured as PI CEO) (41). MD tumor cells have been related to MDV-IS in previous studies (34, 35). However, our results indicate that the late-MDV-IS evaluated in this model is not related to tumors and prove that MDV-IS is even more complex than previously thought. In addition to the early-MDV-IS, there might be two other phases of late-MDV-IS: the one evaluated in this model and unrelated to tumors and the one induced by MD transformed cells. Because the late-MDV-IS evaluated in this study cannot be prevented by MD vaccination (41), it is imperative that further studies are done to develop methods of diagnosis and control.

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5.8 Tables

Table 5.1 Experimental design

Exp[a]	MD challenge[b]			ILTV vaccination and challenge[c]			
	Challenge	**Passage history**	**Dose (pfu)/Age**	**Vaccine[c]**	**Dose/Age[c]**	**Challenge[c]**	**Dose/Age[c]**
1 & 2	-	-	-	-	-	-	-
1 & 2	-	-	-	-	-	Illinois	4000/30d
1 & 2	-	-	-	CEO	MR/15d	Illinois	4000/30d
1	Md5	7 (DEF) + 2 (CKC)	Contact/1d	CEO	MR/15d	Illinois	4000/30d
1	Md5-BAC	5 (DEF)	2000/1d	CEO	MR/15d	Illinois	4000/30d
1	Md5-BACΔMEQ	5 (DEF) + 4 (CEF)	2000/1d	CEO	MR/15d	Illinois	4000/30d
2	686	10 (DEF) + 2 (CKC)	Contact/1d	CEO	MR/15d	Illinois	4000/30d
2	686-BAC	10 (DEF) + 6 (CEF)	2000/1d	CEO	MR/15d	Illinois	4000/30d
2	686-BACΔMEQ	10 (DEF) + 6 (CEF)	2000/1d	CEO	MR/15d	Illinois	4000/30d

[a] Two experiments were conducted, both experiment included three control groups (above thick border line): negative control, LTV control and CEO vaccine control.

[b] Three strains originated from vvMDV strain Md5 were evaluated (Md5, Md5-BAC, Md5-BACΔMeq) in experiment 1. Three strains originated from vv+MDV strain 686 were evaluated (686, 686-BAC and 686-BACΔMeq) in experiment 2. Challenge with original strains (Md5 and 686) was by contact with shedder chickens at day of age (1d). Challenge with molecular clones was done by subcutaneous inoculation of 2,000 PFU of the molecular clone at day of age (1d). Passage history of the viral strain is indicated; DEF= duck embryo fibroblast; CKC= chicken kidney cells; CEF = chicken embryo fibroblasts.

[c] CEO vaccine were used in all experiments at manufacturer's recommendation (MR) at 15 days old via drinking water. ILTV challenge was conducted using virulent strain Illinois strain (4000pfu) at 30 days old via intratracheal inoculation.

Table 5.2: Role of meq on late-MDV-IS and tumors.

Experiment[a]	Treatment[a]	Gross tumor (%)[b]	PI CEO[c]
1	Md5/CEO/ILTV	10.34[A]	94.05 [A]
1	Md5-BAC/CEOI/LTV	13.79 [A]	95.51 [A]
1	Md5-BACΔMEQ/CEO/ILTV	0 [A]	109.1 [A]
2	686/CEO/ILTV	75.61 [A]	45.6* [A]
2	686-BAC/CEO/ILTV	48.83 [B]	84.22 [B]
2	686-BACΔMEQ /CEO/ILTV	0 [C]	89.61 [B]

[a] Two animal experiments was conducted to determine the role of Meq on late-MDV-IS. In experiment 1, three strains derived from vvMDV Md5 were evaluated (Md5, Md5-BAC, Md5-BACΔMeq). In experiment 2, three strains derived from vv+MDV 686 were evaluated (686, 686-BAC and 686-BACΔMeq).

[b] Percentage of chickens that developed gross tumors at the time of death or at the termination of the study (35 days of age). For each experiment, different alphabet indicates statistic significant differences ($p<0.05$).

[c] Protection index of vaccine CEO (PI CEO) in each treatment group. Results are presented in percentage as relative values to the PI CEO of the control group -/CEO/ILTV in their respective experiment. Asterisk (*) indicate that significant differences ($p<0.05$) were found between that group and the control group -/CEO/LTV of the same experiment. For each experiment, different alphabet indicates statistic significant differences ($p<0.05$).

Table 5.3: Tumor frequency in 4 animal experiments and correlation with late- MDV-IS

Experiment[a]	Treatment[b]	Gross tumor (%)[c]	PI CEO[d]	Correlation (PI CEO vs gross tumor)[e]
1	648A/CEO/ILTV	50	31.82	r=-0.162 (p>0.05)
1	HVT/648A/CEO/ILTV	24	76.5	
1	HVT+SB1/648A/CEO/ILTV	4	42	
1	CV1988/648A/CEO/ILTV	13	35.37	
2	GA/CEO/ILTV	11.5	86.96	
2	Md5/CEO/ILTV	10.34	94.05	
2	Md5-BAC/CEO/ILTV	13.79	95.51	
3	617A/CEO/ILTV	48	79.61	
3	Md5/CEO/ILTV	58.33	84.38	
3	$648A_{ATCC}$/CEO/ILTV	86.36	61.28	
3	686/CEO/ILTV	86.36	32.16	
4	686/CEO/ILTV	75.61	45.6	
4	686-BAC/CEO/ILTV	48.83	84.22	
4	HVT (IO)-CVI988(D1)/686/CEO/ILTV	0	53.85	
4	HVT+CVI988 (IO)/686/CEO/ILTV	0	54.81	
4	Md5-BACΔMeq/686/CEO/ILTV	0	95.22	

[a] Study was conducted on using data of four different experiments. Data of 487 chickens were included in the study.

[b] Chickens of 16 treatment groups were analyzed in this study. All chicken were either vaccinated with MDV vaccine and infected with MDV or just infected with MDV prior to CEO vaccination and ILTV challenge.

[c] Gross tumor frequency of each treatment group

[d] CEO protection index is calculated to evaluate the ability of various MD vaccine protocols to protect against late-MDV-IS. Values are expressed as percentage and they are relative to the protective values of the negative control -/CEO/LTV.

[e] Pearson correlation were conducted between frequency of gross tumor and PI CEO of all groups under study. Statistical significance was considered when $p<0.05$.

CHAPTER 6

Study of the pathogenesis of late-Marek's disease virus-induced immunosuppression

6.1 Abstract

Marek's disease virus (MDV) induces immunosuppression in chickens (MDV-IS). We have recently demonstrated that MDV-IS can occur in commercial chickens bearing maternal antibodies (MAb) against MDV and protected by vaccination against the development of tumors. We have named such immunosuppression as late-MDV-IS to differentiate it from the early-MDV-IS associated with lymphoid organ atrophy in chickens lacking MAb. Late-MDV-IS is greatly affected by MDV pathotype as only vv+MDV strains could induce it in a previous study. Recently, we have described that vv+MDV strain 686 but not a molecular clone (686-BAC) or the molecular clone lacking the two copies of the oncogene meq (686-BACΔMEQ) induced late-MDV-IS. The objective of the present study was to identify differences in the pathogenesis of 686, 686-BAC, and 686-BACΔMEQ that could contribute to differences on their immunosuppressive ability (evaluated by the late-MDV-IS-ILT model and by the humoral immune responses against infectious laryngotracheitis virus). Transcription of various MDV genes (ICP4, pp38, gB, and meq), expression of MDV microRNAs (miRNA mdv1-miR-M4-5p and mdv1-miR-M2-3p), and expression of MHC-I antigen in $CD45^+$ spleen cells were evaluated at 36 days following MDV challenge. Major differences in every evaluated feature was found between strain 686-BACΔMEQ and the two oncogenic strains 686 and 686-BAC. Differences between 686 and 686-BAC, however, were more subtle being only remarkable the ability to induce late-MDV-IS in the ILT model (686 induced it and 686-BAC did not). The remaining evaluated features had a similar trend although the effect was more severe in 686. Strain 686 reduced more severely the humoral immune responses to ILTV (1132 *vs.* 2167), induced more tumors (76% *vs.* 45%), have

higher levels of meq transcripts (2.1E+09 *vs.* 4.98E+8) and higher expression of MDV miRNAs (mdv1-miR-M4-5p and mdv1-miR-M2-3p) in the spleen, and reduced further the percentage of $CD45^{+}$-MHC-I^{+} splenocytes (13% *vs.*32%) than molecular clone 686-BAC. The results of this study show a progression of virulence from the least virulent strain (686-BACΔMEQ) to the most virulent strain (686); being strain 686-BAC of intermediate virulence. In this study, virulence was observe not only by the ability to induce tumors but also by the ability to induce late-MDV-IS. ILT model can detect MDV-IS once it becomes clinical and such threshold was only bypassed by the most immunosuppressive MDV strain (686).

6.2 Introduction

Marek's disease (MD), caused by Marek's disease virus (MDV) is a complex lymphoproliferative disease of chickens affecting the poultry industry worldwide. The most generalized hypothesis for the pathogenesis of MDV was described by Calnek (1) and it is commonly known as the Cornell theory. This theory identifies four phases in the MDV pathogenesis: 1) early cytolytic infection primarily in B cells that occurs between 3 and 5 days post infection, 2) latent infection of T lymphocytes, 3) reactivation from lymphocytes or secondary cytolytic infection, and 4) transformation of lymphoid cells leading to formation of tumor (1). In addition, MDV is able to induce immunosuppression (MDV-IS) at various times during MDV pathogenesis. Schat et al. recognized two phases of MDV-IS: early and late (2). Early-MDV-IS is associated with the early replication of MDV in the lymphoid organs. It occurs only in chickens unvaccinated against MD and that lack MAb against MDV, having little or no relevance in commercial poultry (3, 4). Late-MDV-IS is associated

with immune dysregulation occurring during the stages of latency and transformation. Previous studies have shown that late-MDV-IS occurs in chickens regardless of vaccination and MAb status (5) and does not seem to be related to the development of MD tumors (6).

Late-MDV-IS pathogenesis is poorly understood. MDV can dysregulate the immune system by various mechanisms. It regulates the expression of MHC-I (12, 13) and MHC-II (14); being this feature more remarkable in vv+MDVs. Likewise, vv+MDV induces significantly higher level of NO than less virulent strains and that could lead to NO-induced apoptosis (15). Recently, Bernberg*, et al.* (16) suggested that overexpression of mdv-miR-M4 in HVT resulted in reduction of commercial vaccine efficiency against *Salmonella Enteritidis*. In addition, several studies have demonstrated that MDV-transformed cells can induce immunosuppression. MD tumors expresses chicken fetal antigen that interfere with NK killer cells activity and CD30 antigen that may aid in the switch of immune responses toward Th2 response (17, 18). Moreover, addition of MDV lymphoblastoid cells to normal spleen cells was shown to inhibit the proliferation response to mitogen (19).

In a previous work, we have developed a model to study late-MDV-IS under laboratory conditions. This model indirectly evaluates late-MDV-IS by assessing the effect of vv+MDV infection on the efficacy of infectious laryngotracheitis (ILT) vaccination in commercial meat type chickens (20). Our study has shown that late-MDV-IS can happen in commercial chickens bearing MAb against MDV that did not have lymphoid organ atrophy and that are protected by vaccination against the development of tumors (5). In addition, we have also demonstrated that only the most virulent strains of MDV (vv+) can induce MDV-

IS in the ILT model (21). In a recent study, we have demonstrated that vv+MDV strain 686 but not a molecular clone (686-BAC) or the molecular clone lacking the two copies of the oncogene meq (686-BACΔMEQ) induced late-MDV-IS. Strain 686 is the most virulent MDV ever isolated in USA (22), molecular clone 686-BAC was derived from it but it has been reported to be of lessen virulence (23). Recombinant strain 686-BACΔMEQ did not induce either tumors or late-MDV-IS in our previous study. The objective of the present study was to identify differences in the pathogenesis (viral gene expression and host immune responses) of 686, 686-BAC, and 686-BACΔMEQ that could contribute to differences on their immunosuppressive ability. The immunosuppressive ability of the three MDV strains was evaluated using the model late-MDV-IS-ILT model (20). In addition, the effect of MDV on the humoral immune responses against infectious laryngotracheitis virus (ILTV) was also measured.

6.3 Materials and Methods

Chickens. Commercial specific pathogen free SPAFAS chickens (Charles River SPAFAS, N Franklin, CT) were used as MDV shedder chickens. Female commercial meat type chickens bearing MAb against MDV were used as experimental chickens.

Viruses and vaccine. Turkey herpesvirus (HVT) FC-126 strain (Merial Select, Inc., Gainesville, GA) were used to vaccinate shedder chickens at 18 days of embryonation. Serotype 1 MDV strain, 686 (vv+) at passage 10 in duck embryo fibroblasts (DEF) and 2 passages in chicken kidney cells (CKC) (24), 686-BAC at 10 passages in DEF and 6 passages in CEF (23) and 686-BACΔMEQ (Reddy et al., unpublished) at 10 passages in DEF

and 6 passages in CEF were used as MDV challenge. Chicken embryo origin (CEO) vaccine strain of ILTV was obtained from a commercial manufacturer (Merial Select, Inc., Gainesville, GA, USA. Illinois-N71851 strain of ILTV that has been characterized as a virulent ILTV strain were used for ILT challenge (25).

Experimental Design. An animal experiment was conducted following the guidance and under approval of North Carolina State University Institutional Animal Care & Use Committee (IACUC). The experimental design of the late-MDV-IS ILT model (20) was used. Briefly, SPAFAS chickens were vaccinated with HVT vaccine *in ovo* via amniotic route at 18 days of embryonation (ED) to avoid the development of transient paralysis and to ensure survival. At hatch, shedder chickens were infected with 2000 PFU of vv+MDV 686 strain subcutaneously and maintained in isolation for 15 days prior to the beginning of experiment. Experimental chickens housed with the shedders were tested by real time PCR for both MDV and HVT at 7 days and at the end of the experiment. Horizontal transmission of MDV was confirmed by detecting MDV DNA in the feather pulp of experimental chickens at 7 days of age. Horizontal transmission of HVT from the shedders to the experimental chickens did not occur.

Newly hatched meat type experimental chickens were wing banded, divided into groups (42 chickens per group) and placed in environment controlled room (BSL-2) for the rest of the experiment. Details of the experiment are shown in Table 6.1. The experiment included three control groups 1) Group 1 received no treatment; 2) Group 2 was challenged with ILTV; 3) Group 3 was vaccinated with CEO and challenged with ILTV. In addition,

there were three MDV challenged groups (Groups 4, 5, and 6) as follows: 4) Group 4 was challenged with 686 by contact at day of age, vaccinated with CEO, and challenged with ILTV; 5) Group 5 was infected with 2,000 PFU of 686-BAC via subcutaneous injection at day of age, vaccinated with CEO, and challenged with ILTV; and 6) Group 6 was infected with 2,000 PFU of 686-BACΔMEQ via subcutaneous injection at day of age, vaccinated with CEO, and challenged with ILTV.

When meat type chickens were 15 days of age, they were vaccinated with CEO vaccine via drinking water per manufacturer's recommendations. Two weeks later (day 30 of the experiment), chickens were challenged intratracheally with 2000 PFU of ILTV. At this time all chickens were identified with colored leg bands. Every chicken was monitored daily for six days between challenge with ILTV and the termination of the experiment to evaluate if clinical signs of ILT were present. All chickens were necropsied at termination (day 7 after ILTV challenge) or after death, and evaluated for gross lesions consistent with ILT and MD. In addition, blood and feather pulp samples were collected from every chicken at termination; and spleens were collected from six chickens per treatment group for nucleic acid extraction and flow cytometry analysis at day six post LTV challenge.

Clinical Signs. Each bird was identified and observed daily for clinical signs of ILT such as gasping, coughing, sneezing, conjunctivitis and expectoration. Mortality was also recorded.

Development of ILT clinical sings (CS) was assessed by ILT-CS score as previously described (20). Briefly, ILT-CS score was calculated as the total number of days a particular chicken showed ILT clinical signs (range from 0 to 6).

Gross Lesions. Upon necropsy, dead birds were evaluated for ILT and MD lesions. ILT gross lesions (GL) were scored from 0 to 4 in a gradient of severity. The criteria was as follow: 0 = normal, 1 = presence of light mucus, 2 = congested, thick mucus or bloody mucus, 3 = presence of caseous exudate, and 4 = tracheal plugs.

MD lesions were observed by evaluating presence of tumors in nerves and viscera as well as MD lesions in eye and skin.

Assessment of late-MDV-IS. Evaluation of late-MDV-IS was done based on ILT clinical signs (CS) and gross lesions (GL) as previously described (20). Number of days exhibiting CS was calculated for each chicken (values 0-6). GL in trachea at termination were scored based on severity (values 0-4). ILT index was calculated to assess severity of the disease based on CS and GL.

ILT index (ILTI) = CS + GL (values 0-10)

Protection index of the CEO vaccine (PIO CEO) was calculated using ILTI values from the positive control group (-/ILTV) and the CEO vaccinated groups (-/CEO/ILTV, and MDV/CEO/ILTV) to measure protection provided by CEO vaccine.

Protection index (PI CEO) = $(ILTI_{-/ILTV} - ILTI_{TXT}) / ILTI_{-/ILTV}) \times 100$

$ILTI_{-/ILTV}$ is ILT index of -/-/ILTV control group.

$ILTI_{TXT}$ is ILT index of any treatment group that had been vaccinated with CEO vaccine.

Results are presented as PI CEO of the MDV challenged groups (MD vaccinated or unvaccinated) relative to the value of the PI CEO of the control group (None/None/CEO/ILTV).

Assessment of MDV-induced tumors. MDV-induced tumors were evaluated by gross inspection during necropsy at the termination of the experiment or when the chickens died. In addition, early diagnosis using real time PCR was conducted as described (26-28). Briefly, oncogenic MDV DNA load was evaluated by real time PCR in feather pulp from every chicken at the termination of the experiment. Ct ratio was calculated for each sample by dividing the Ct value of housekeeping gene (GAPDH) by the Ct value of the MDV gene (gB) amplified. Based on the Ct ratio, sample were classified as negative (GAPDH gene amplified but the MDV gene did not); latently infected (Ct ratio is lower than the threshold level 1.7); neoplastically transformed (Ct ratio is equal or higher than the threshold level 1.7) (28, 29).

Real time PCR. DNA was extracted from feather pulp and spleen using ArchivePure DNA tissue kit (5-Prime Inc, Gaithersburg, Maryland, USA) following manufacturer's recommendations.

DNA samples were amplified with three pairs of primers specific for the glycoprotein B (gB) gene of serotype 1 MDV, MDV EcoRI Q fragment of serotype 1 MDV strains (Meq) and for the chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Sequence for the respective forward and reverse primers are listed in Table 6.2. Amplifications were done using an Mx3005 Stratagene thermocycler (Stratagene, La Jolla, CA) in a 25-ul PCR reaction containing 50 ng of DNA, 0.2 μM of each primer, and SYBR® Green PCR master

mix (Brilliant® SYBR® Green, Biocrest-Stratagene, Cedar Creek, TX). The reaction was cycled 50 times at 95°C denaturation for 15 sec and a 60°C combined annealing/extension for 60 sec. Fluorescence was acquired at the end of the annealing/extension phase. The melting curves were obtained at the end of amplification by cooling the sample at 20°C/sec to 60°C and then increasing the temperature to 95°C at 0.1°C/sec. The parameter threshold cycle (Ct) was calculated for each PCR reaction by establishing a fixed threshold. Ct is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Relative quantification of the amount of target in unknown samples was accomplished by the comparative Ct method. Ct ratios were established for each sample (Ct ratio GAPDH-gB= Ct GAPDH/Ct gB). The higher the Ct ratio, the higher the load of MDV (26, 29).

Real time RT-PCR. RNA was extracted from spleen samples using the Perfect Pure RNA tissue kit (5-Prime Inc, Gaithersburg, Maryland, USA) following manufacturer's recommendation. Real time reverse transcriptase PCR (real time RT-PCR) was performed to measure transcription of MDV ICP4, pp38, gB and Meq genes. Primers sequence are listed in Table 6.2. Housekeeping gene 28S rRNA were used as internal control (30). Amplifications were done in an Mx3005 Stratagene thermocycler (Stratagene, La Jolla, California, USA) by amplifying 25µl reactions using Brilliant II SYBR® Green Q-PCR Master Mix (Agilent Technologies, Santa Clara, CA, USA). The profile cycles used were 1 cycle of 50°C for 30 min; 1 cycle of 95°C for 10 min; 50 cycles of 95°C for 10 sec and 56°C for 1 min; and 1 cycle of 95°C for 1 min, 55°C for 30 sec, and 95°C for 30 sec. The melting curves were obtained at the end of amplification by cooling the sample at 2.0^{o}C/s to 60^{o}C and then increasing the temperature to 95^{o}C at 0.1^{o}C/s. Samples were ran in duplicates and the Ct

was averaged. Average Ct value were subtracted from total number of cycles (50 - average Ct). Correction for differences in RNA levels between samples was done by using a 28S correction factor via the formula: (50 - overall mean Ct 28S rRNA-specific product from all samples) / (50 - average Ct for 28S rRNA-specific product for each sample). Corrected viral gene means was calculated using the formula: 28S correction factor X (50 - Ct value for viral gene) X viral gene slope/28S gene slope) (31).

Analysis of microRNA expression by real-time PCR. First strand complementary DNA (cDNA) was synthesized from 1μg of polyadenylated total RNA from each spleen samples using a miRNA 1st strand cDNA synthesis kit (Agilent, Santa Clara, CA) following the manufacturer's instructions. For miRNA real time PCR, each reaction contained 1 μl of cDNA and 24 μl of High-specificity miRNA QPCR Core Reagent Kit Master Mix (Agilent Technologies, Santa Clara, CA, USA). PCR was performed using the following conditions: 95°C for 15 min followed by 40 cycles of [95°C 10 sec and 60°C 20 sec] using Mx3005 Stratagene thermocycler (Stratagene, La Jolla, California, USA). Expression of MDV miRNA mdv1-miR-M4-5p, mdv1-miR-M2-3p and chicken snoU83B was conducted using forward primers (mature miRNA sequence) previously reported and they are listed in Table 6.2 (32). Average Ct value were subtracted from total number of cycles (40 - average Ct). Correction for differences in miRNA levels between samples was done by using a snoU83B correction factor via the formula: (40 - overall mean Ct snoU83B miRNA-specific product from all samples) / (40 - average Ct for snoU83B miRNA product for each sample). Corrected viral gene means was calculated using the formula: snoU83B correction factor X (40 Ct value for viral gene) X viral gene slope/ snoU83B gene slope).

Serology. Serum samples were analyzed for antibody titers against ILTV using the commercial ProFLOK® Fowl Laryngotracheitis Virus Antibody Test Kit (Synbiotic Corporation, Kansas City, MO). The test was performed following the manufacturer's recommendations.

Flow cytometry analysis. Spleen samples were collected in Leibovitz-McCoy media (Sigma-Aldrich, Inc, St. Louis, Missouri, USA) upon necropsy. Collected spleen were forced through sterile gauze and allowed to sediment for few minutes. Cell suspension were carefully separated from tissue debris and washed via centrifugation at 1000 rpm for 10 minutes at 4°C. Cells were then frozen in freezing medium (55% Leibovitz-McCoy medium, 25% calf serum and 20% DMSO) and kept it liquid nitrogen until used for analysis. For analysis, frozen cells were thawed, washed twice, and then suspended in cold flow cytometry media. Cells ($1x10^6$) were incubated with the appropriate dilution of primary monoclonal antibody for 30 min at room temperature. The excess primary antibody was removed by three successive washes in cold flow cytometry media. Finally, cells were incubated in DAPI stain for 15 minutes prior to flow cytometry analysis. Twenty thousand cells were analyzed by flow cytometry using BD LSR II (BD Biosciences, San Jose, CA, USA).

Antibodies

All antibodies used in this study were purchased from SouthernBiotech (Birmingham, AL, USA) either as fluorescein isothiocynate conjugated or phycoerythrin conjugated. Chicken antigens CD45 and major histocompatibility complex (MHC) antigen class I were detected by monoclonal antibodies CD45 (33) and F21-2 (34), respectively.

Data analysis and statistics. Data were analyzed with statistical program Statistica (Stat Soft, Tulsa, Oklahoma, USA) and SPSS (IBM, New York, USA). Comparison between two groups were conducted using Student's t test while comparison between more than two groups were conducted using one-way analysis of variance (ANOVA) test. The Scheffe test was used as a post hoc analysis.

6.4 Results

Immunosuppressive ability of strains derived from 686 (686, 686-BAC, and 686-BACΔMEQ). Strain 686 but not 686-BAC and 686-BACΔMEQ induced late-MDV-IS in the ILT model (Figure 6.1 A). Results are presented as the PI CEO of each group relative to the PI CEO of the control group None/None/CEO/ILTV (baseline of 100%). PI CEO of group 686/CEO/ILTV was significantly lower (46%) than that of the control group None/CEO/ILTV (100%). No significant differences were found between group 686-BAC/CEO/ILTV (84%), 686-BACΔMEQ/CEO/ILTV (89%), and the control group None/CEO/ILTV (100%).

The effect of the three evaluated MDV strains on the humoral responses against ILTV was evaluated and is presented in Figure 6.1B. Chickens in the control group -/-/CEO/ILTV showed high level of antibody titer (3060) and it was significantly reduced in the group challenged with strain 686 (1132). Reduction on the antibody titers was observed in the group challenged with 686-BAC (2167), albeit not significant ($p>0.05$). Chickens challenged with strain 686-BACΔMEQ had no reduction of antibody titers (3416) against ILTV.

Neoplastic ability of strains derived from 686 (686, 686-BAC, and 686-BACΔMEQ). Evaluation of gross tumors at the death of the chickens or at the termination of the study (35 days of age) was conducted and results are presented in Figure 6.2A. None of the chickens inoculated with strain 686-BACΔMEQ developed tumors. Chickens challenged with strain 686 developed the highest frequency of tumors (76%). Significantly lower ($p<0.05$) percentage of chickens (45%) inoculated with 686-BAC developed tumors.

Early diagnosis of MD evaluated by real time PCR in feather pulp samples rendered similar results as those obtained by gross inspection of tumors (Figure 6.2B). None of the chickens inoculated with strain 686-BACΔMEQ had levels of MDV DNA compatible with tumors. Chickens challenged with strain 686 had 92% of the chickens with high levels of MDV DNA in the feather pulp compatible with tumors. Significantly lower ($p<0.05$) percentage of chickens (44%) inoculated with 686-BAC had high levels of MDV DNA in the feather pulp compatible with tumors.

MDV gene transcriptions and expression of MDV microRNAs. Detection of oncogenic MDV DNA as well as expression of various MDV transcripts were evaluated in the spleen of 6 chickens per treatment group at the termination of the experiment. Results are presented in Table 6.3. MDV DNA (gB and Meq) was detected in 100% of the spleens (6 out of 6) of chickens challenged with 686 and 686-BAC. MDV DNA (gB) was detected in 67% of the spleens of chickens challenged with 686-BACΔMEQ. Regardless of the presence of the viral DNA, neither pp38, gB and meq transcripts nor expression of mdv1-miR- M4-5p was detected in the spleens of chickens infected with 686-BACΔMEQ; only low amount of ICP4 transcripts and minimum expression of mdv1-miR-M2-3p were detected. By contrast

chickens challenged with 686 or 686-BAC had very high levels of MDV gene transcripts and high expression of MDV microRNAs. In all cases, levels were higher in the chickens challenged with 686 than in those challenged with 686-BAC but differences were not significant ($p>0.05$).

Expression of MHC-I in splenocytes. Expression of MHC-I in splenocytes was evaluated and results are presented in Figure 6.3. Results are presented relative to the values of chickens in the control group None/CEO/ILTV. Frequency of $CD45^+$-$MHC\text{-}I^+$, $MHC\text{-}I^+$, and CD45+ cells was lower in group 686/CEO/ILTV than in the control None/CEO/ILTV group. No significant differences in the frequency of $CD45^+$-$MHC\text{-}I^+$ and $MHC\text{-}I^+$ were detected between groups 686/CEO/ILTV and 686-BAC/CEO/ILTV. However, there was a greater reduction of $CD45^+$ cells in group 686/CEO/ILTV than in group 686-BAC/CEO/ILTV. Group 686-BACΔMEQ group did not affect the frequency of any of the evaluated immunophenotypes.

6.5 Discussion

A model to evaluate late-MDV-IS under laboratory conditions has been recently developed (20). In a previous work, we have used that model to demonstrate that strain 686 but not the molecular clone 686-BAC derived from it or the recombinant 686-BAC induced late-MDV-IS. In the present work, we have further evaluated the pathogenesis of these three related strains to identify features that might be related to late-MDV-IS. Our results demonstrated that deletion of meq resulted in total abrogation of neoplastic and immunosuppressive abilities, and reduced or knockout MDV gene transcription and expressions of MDV miRNAs. Furthermore strain 686-BACΔMEQ did not affect the

percentage of $CD45^+$, MHC-I^+, or $CD45^+$-MHC-I^+ in the spleen. By contrast, molecular clone 686-BAC, although it did not induce late-MDV-IS in the ILT model, reduced humoral immune responses against ILTV, reduced the percentage of MHC-I^+ cells in the spleen, was able to induce tumors in 45% of the chickens, and had high levels of viral transcripts and high expression of viral miRNA in the spleens. Strain 686 was the only strain that induced late-MDV-IS in the ILT model. It also reduced greatly humoral immune responses against ILTV, and the percentage of $CD45^+$, MHC-I^+, or $CD45^+$-MHC-I^+ in the spleen. Furthermore, chickens inoculated with 686 suffered the highest rate of tumors and had the highest levels of MDV gene transcripts and expression of viral miRNA. The results of this study show a progression of virulence from the least virulent strain (686-BACΔMEQ) to the most virulent strain (686); being strain 686-BAC of intermediate virulence. In this study, virulence was observe not only by the ability to induce tumors but also by the ability to induce late-MDV-IS.

In our study, 686-BAC induced significant lower frequency of tumors than the wild type strain 686. Reddy et al. (23) reported that 686-BAC had lower virulence than 686 and suggested that such reduction on pathogenicity could be due to loss of US2 and/or presence of mini F plasmid sequence. They also indicated that differences could be due to the selection of one subpopulation of virus of lower virulence while the process of cloning. The lower virulence of 686-BAC was later demonstrated by Dunn (personal communication) by the pathotyping assay; while both 686 and 686-BAC typed within the vv+ pathotype, 686 virulence rank was higher than 686-BAC. Our results confirm that 686-BAC is indeed less virulent not only in relation to its ability to induce tumors but also to induce late-MDV-IS,

confirming that the latter is a unique feature of the most virulent MDVs as we have reported previously (21).

The virulence trend observed in virulence (tumors and late-MDV-IS) could be observed as well when evaluating MDV transcripts and viral miRNA expression. The most virulent strain 686 had the highest levels of all MDV transcripts and viral miRNA, although differences with strain 686-BAC were not statistically significant. Clear differences, however were detected in chickens inoculated with strain 686-BACΔMEQ. Deletion of MEQ resulted in marked reduction of immediate early gene ICP4 transcripts and total abrogation of transcriptions of early gene pp38, oncogene meq, and late gene gB. Furthermore deletion of meq resulted in lack of expression of mdv1-miR- M4-5p and severe reduction of mdv1-miR-M2-3p. Our results suggest that deletion of meq completely disrupt the ability of 686-BACΔMEQ to reactivate from latency in lymphoid cells. It has been shown that meq deletion does not affect early replication in lymphoid organs (35), but severely reduces the amount of viruses that could be isolated from blood at 2 weeks of age (35). In this study, we have demonstrated that although 686-BACΔMEQ genome was present in splenocytes, the ability of the virus to reactivate from latency in lymphocytes is hampered. Reactivation from latency is considered to be necessary for the development of tumors (36, 37), and therefore this might explain the lack of neoplastic ability of this virus. The effect of meq deletion on the ability of the virus to replicate seems to affect not only to the meq-deleted virus but also to other oncogenic MDVs in chickens co-infected with both meq-deleted virus and oncogenic complete MDVs. In a previous study, we have demonstrated that vaccine Md5-BACΔMEQ severely reduced replication of the oncogenic virus 686 (5). Interestingly, Md5-BACΔMEQ

is the only vaccine able to control against both MDV-induced tumors and late-MDV-IS. Further studies to better understand how lack of meq can regulate gene expression not only in the meq-deleted virus but in other MDVs present in the same chicken are warranted.

It has previously been shown that MDV down-regulates cell surface expression of MHC-I protein during active but not latent infection of chicken cells (12). Yu et al. (38) reported that MHC-I and β2 microglobulin transcripts were up-regulated during early infection but down-regulated at 14 and 28 days post infection. In an in vivo experiment, Gimeno et al. (13) showed that the vv+MDV strain 648A but not vMDV strain GA induced severe down-regulation of MHC-I expression on endothelial cells and in mononuclear cells infiltrating the brain at 19-26 dpi. Our results show that vv+ strain 686 significantly decreased the percentage of cells expressing MHC-I in the spleen, including the percentage of $CD45^+$ cells expressing MHC-I ($CD45^+$-MHC-I^+) at 36 dpi. Since the percentage of $CD45^+$ cells was also reduced it is unknown if 686 down-regulated MHC-I and further studies are warranted. Down-regulation of MHC-I in $CD45^+$ splenocytes could hamper cell mediated immune responses and it might be involved in the late-MDV-IS detected in the ILT model. The late-MDV-IS ILT model evaluates immunosuppression affecting cell mediated immune responses as this is the base for protection of ILT (39). The late-MDV-IS ILT model is capable of detecting those strains that can induce clinical immunosuppression. In this study, the only strain able to induce late-MDV-IS was the most virulent 686. Equally in previous studies only the most virulent strains 686 and 648A were able to induce late-MDV-IS (21). Molecular clone 686-BAC failed to induce late-MDV-IS in the ILT model but it reduced the frequency of cells expressing MHC-I ($P<0.05$) when compared to the control

group -/CEO/ILTV. Strain 686-BAC also reduced the frequency of $CD45^+$ cells expressing MHC-I albeit such differences were not significant ($P<0.05$). It is likely that only the most virulent strains are able to reduce the immune responses on the chickens behind a threshold level that can be clinically detected (late-MDV-IS ILT model). However, MDV strains of lower virulence such as strain 686-BAC, although inducing subclinical immunosuppression in the chickens, could not overpass that threshold. The effect of MDV strains of various pathotypes on MHC-I expression should be evaluated in future experiments to confirm that this is the case.

Inhibition of MHC I antigen processing and presentation pathway is a common mechanism used by herpesviruses to counter the host immune response. Several strategies was used by these viruses to interfere with MHC I pathway such as interference with proteasome activity (40, 41), inhibition of transporter associated antigen processing (TAP) mediated transport (42, 43), prevention of tapasin mediated peptide-loading (44, 45) and virus-mediated endocytosis of MHC I (46). The mechanism by which MDV interfere with MHC-I expression is poorly understood and further studies are warranted.

The ability of MDV to affect the immune responses affect not only cell mediated immune responses (evaluated by the late-MDV-IS ILT model) but also humoral immune responses (9, 21, 47, 48). In this study, we have confirmed that strain 686 reduces the humoral immune responses against ILTV infection. A reduction in antibody titers against ILTV, although not significant, was detected also in chickens inoculated with 686-BAC strain. Deletion of MEQ, however, reverted the negative effect of 686. The effect of meq deletion on the humoral responses has been previously reported (49). These results increase

the spectrum of diseases that might be negatively affected by the late-MDV-IS in commercial poultry and the economic impact of this newly acquired feature of the most virulent vv+MDV strains.

This study show that the ability of MDV to induce IS follow a continuous spectrum and only the most virulent MDVs can overpass a certain threshold level and become clinical in the late-MDV-IS ILT model. Minimum attenuation (as it is the case between 686 and 686-BAC) resulted in decreased IS ability below the threshold level and thus 686-BAC did not induce late-MDV-IS. The ability to induce late-MDV-IS in the ILT model could be very helpful to further characterize newly emerging MDVs. Furthermore, it might help us understanding the evolution of MDV towards more virulence. Down-regulation of MHC-I could be involved in the development of late-MDV-IS and requires further studies. The effect of meq deletion on the pathogenesis of late-MDV-IS also requires additional attention. The fact that meq deletion abrogated reactivation of MDV from latency in lymphocytes might be a critical point in the control of tumors and of late-MDV-IS. This study open new questions on the role of meq in the pathogenesis of MDV and set the basis for further studies.

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6.8 Figures and tables

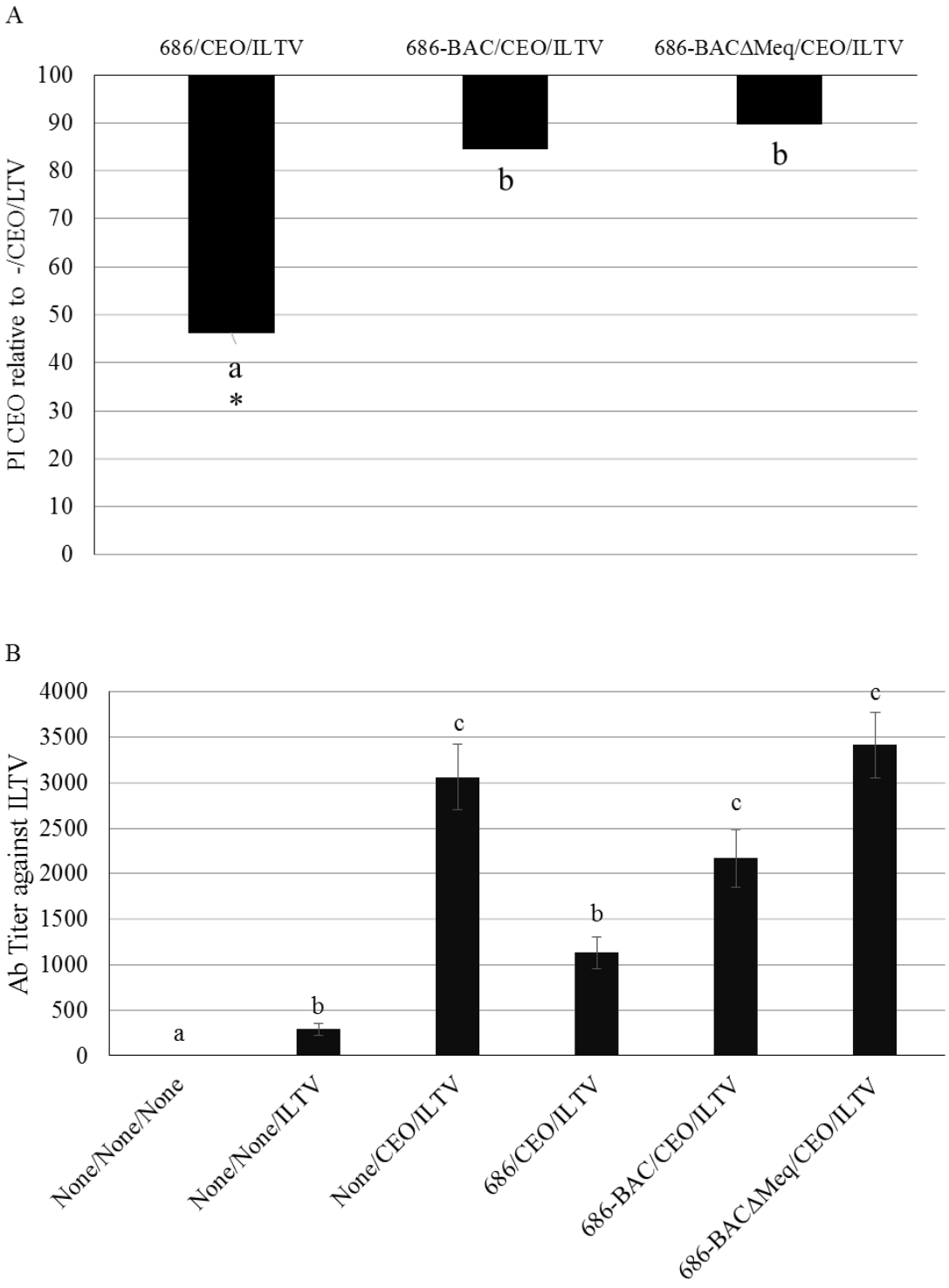
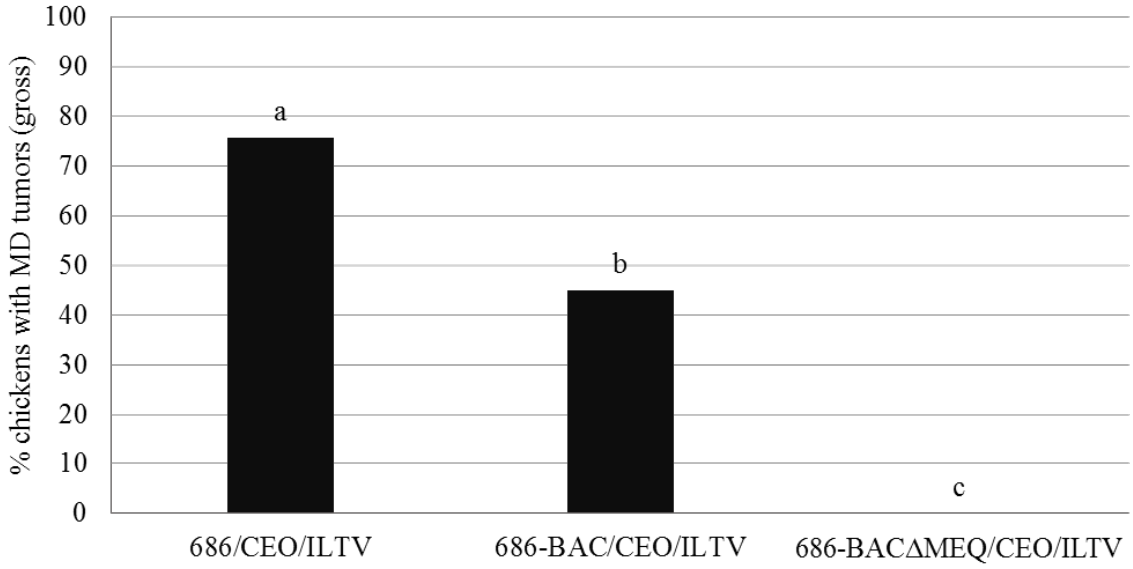


Figure 6.1. (A) CEO protection index (PI CEO) in group 686/CEO/ILTV, 686-BAC/CEO/ILTV and 686-BACΔMEQ/CEO/ILTV). Results are presented as PI CEO values of the treatment group relative to the PI CEO values of the control group None/CEO/ILTV (100% protection). Asterisk (*) under the bars indicate that differences were statistically significant ($p<0.05$) between treatment group and control None/CEO/ILTV. Different letters below the bars indicates that differences among treatment groups were statistically significant ($p<0.05$) **(B)** Antibody titer against ILTV were evaluated using commercial ELISA kit. Different alphabet on top of bars indicate that differences among treatment groups were statistically significant ($p<0.05$).

A


% chickens with MD tumors (gross)
100
90
80
70
60
50
40
30
20
10
0
a
b
c
686/CEO/ILTV
686-BAC/CEO/ILTV
686-BACΔMEQ/CEO/ILTV

B

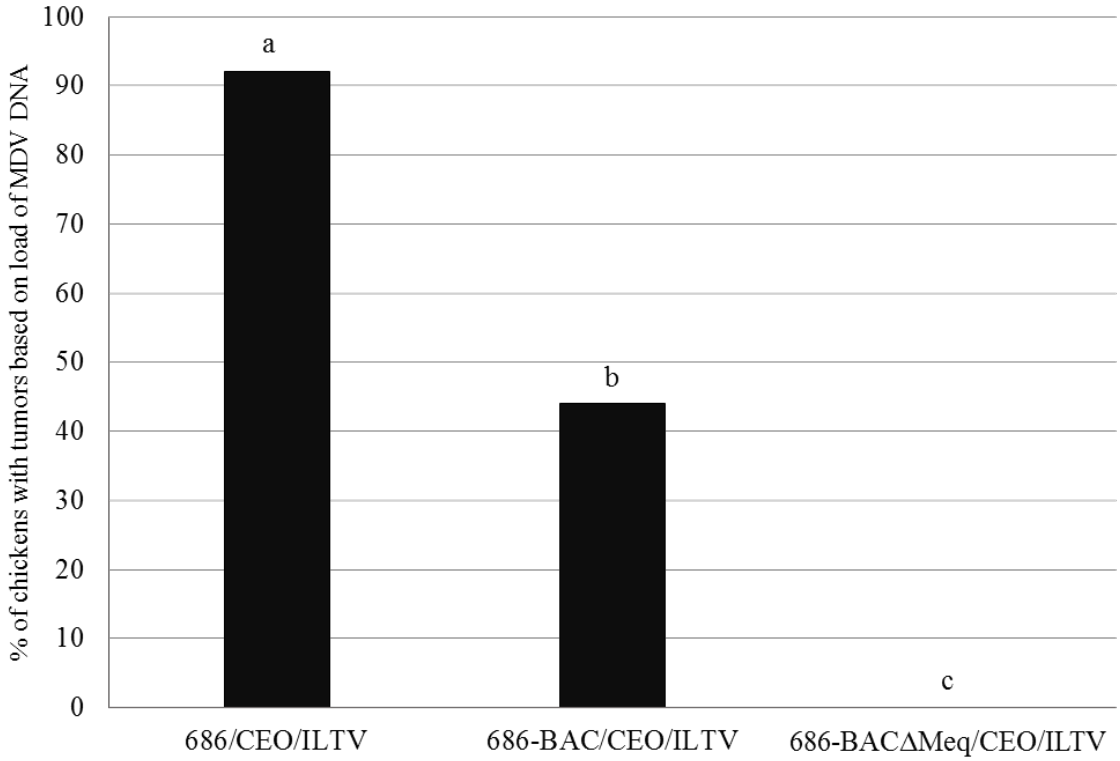

% of chickens with tumors based on load of MDV DNA
100
90
80
70
60
50
40
30
20
10
0
a
b
c
686/CEO/ILTV
686-BAC/CEO/ILTV
686-BACΔMeq/CEO/ILTV

Figure 6.2. (A) Frequency of chickens that develop MDV-induced tumors by the end of the experiment (37 days) following inoculation with different MDV strains (686, 686-BAC and 686-BACΔMEQ. Different alphabet on top of bar indicate that differences were statistically significant ($p<0.05$). **(B)** Percentage of animals with high load of MDV DNA in the feather pulp (compatible with tumor levels) evaluated by real time PCR. Different alphabet on top of bars indicate that differences were statistically significant ($p<0.05$).

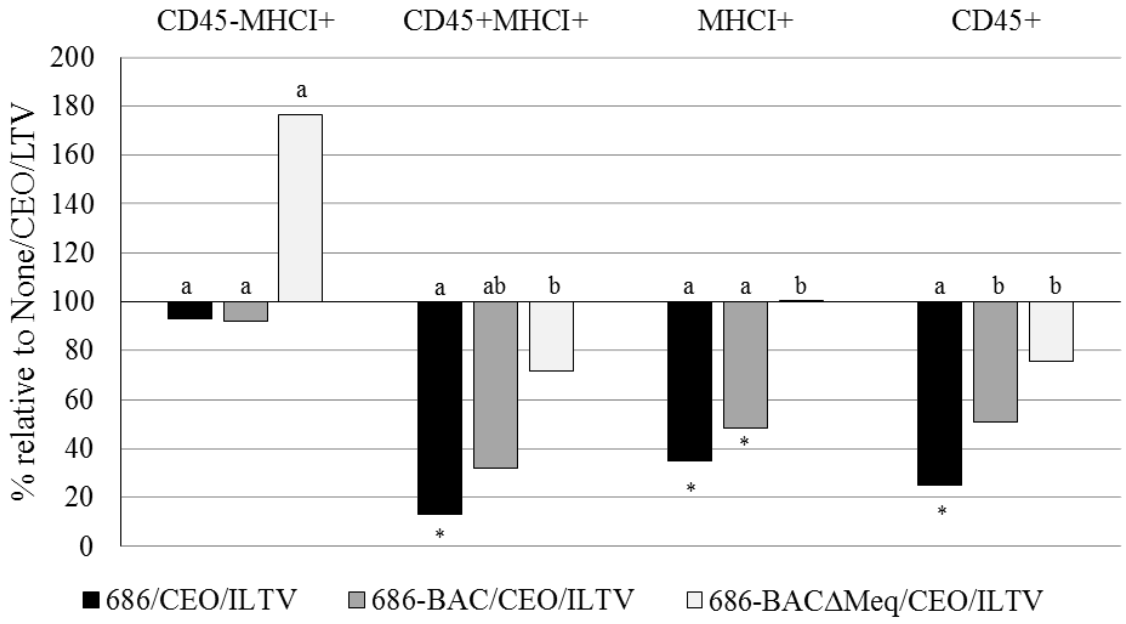


Figure 6.3. Frequency of splenocytes expressing MHC-I and CD45 antigens. Result shows different percentages of cells expressing MHC-I and CD45 antigen in splenocytes of chickens 37 days following inoculation with different MDV strains (686, 686-BAC and 686-BACΔMEQ. Result are expressed relative to the values in the control group None/CEO/ILTV group. There were 6 spleen samples per treatment group. Asterisk (*) under the bars indicate that differences were statistically significant ($p<0.05$) compared to control group None/CEO/ILTV. Different letters on top of bar indicate that differences among treatment groups were statistically significant ($p<0.05$).

Table 6.1. Experimental design

	MD challenge		ILTV vaccination and challenge			
Group[1]	**Challenge[2]**	**Dose (pfu)/Age[2]**	**Vaccine[3]**	**Dose/Age[3]**	**Challenge[4]**	**Dose/Age[4]**
1	-	-	-	-	-	-
2	-	-	-	-	Illinois	4000/28d
3	-	-	CEO	MR/14d	Illinois	4000/28d
4	686	Contact/1d	CEO	MR/14d	Illinois	4000/280d
5	686-BAC	2000/1d	CEO	MR/14d	Illinois	4000/28d
6	686-BACΔMEQ	2000/1d	CEO	MR/14d	Illinois	4000/28d

[1] Experimental chickens were divided into 6 treatment groups. All chickens (42 chickens) were housed in same environmental condition.

[2] Three different strains of MDV were evaluated (686, 686-BAC and 686-BACΔMEQ) which were infected by contact or by inoculation of 2000 PFU via subcutaneous at 1 day.

[3] CEO vaccine were used in all experiments at manufacturer's recommendation (MR) at 15 days old via drinking water.

[4] ILTV challenge were conducted using virulent strain Illinois strain (4000 PFU) at 30 days old via intratracheal inoculation.

Table 6.2. Primers used for real time PCR

Target gene	Sequence	Orientation
GAPDH	5'-GGAGTCAACGGATTTGGCC-3'	Forward
	5'-TTTGCCAGAGAGGACGGC-3'	Reverse
gB	5'-CGGTGGCTTTTCTAGGTTCG-3'	Forward
	5'-CCAGTGGGTTCAACCGTGA-3'	Reverse
Meq	5’-GGTCTGGTGGTTTCCAGGTGA-3’	Forward
	5’-GCATAGACGATGTGCTGCTGA-3’	Reverse
ICP4	5'-CGCCACACGAGAACACAATG-3'	Forward
	5'-GGTTGGAGTAGAGCTGCAACTGT-3'	Reverse
pp38	5'-GTGATGGGAAGGCGATAGAA-3'	Forward
	5'-AGCTACCCCTTTCGGTTTGT-3'	Reverse
28s	5′-GGCGAAGCCAGAGGAAACT-3′	Forward
	5′-GACGACCGATTTGCACGTC-3′	Reverse
mdv1-miR-M4-5p	5’- TTAATGCTGTATCGGAACCCTTC-3’	Forward
mdv1-miR-M2-3p	5’- CGGACTGCCGCAGAATAGCTT-3’	Forward
snoU83B	5’- GTTCGGTGATGAAACCATGGA -3’	Forward

Table 6.3. Expression of various viral transcript in chickens of different treatment group

Treatment	% chickens positive for MDV DNA in spleen[a]		MDV transcript (fold change relative to control) [b]					
	gB	Meq	ICP4	pp38	gB	Meq	mdv1-miR-M4-5p	mdv1-miR-M2-3p
686/CEO/ILTV	100	100	3.59E+07[a]	1.94E+07[a]	3.79E+06[a]	2.10E+09[a]	1.36E+15[a]	6.26E+10[a]
686-BAC/CEO/ILTV	100	100	1.48E+07[a]	7.74E+06[a]	8.69E+05[a]	4.98E+08[a]	4.79E+12[a]	4.67E+09[a]
686-BACΔMEQ/CEO/ILTV	67	0	5.62E+01[b]	1.00E+00[b]	1.00E+00[b]	1.00E+00[b]	1.00E+00[b]	5.87E+00[b]

[a] Percentage of chickens (6 per treatment group) that had detectable levels of MDV genes gB and Meq by real time PCR at the termination of the study.

[b] MDV transcripts in the spleens (6 chickens per treatment group). Results are presented as fold difference relative to control group (None/None/None/None).

Comparison of different expression of MDV transcript between each group are indicated by superscript capital letters; the same letter indicates that no statistically significant differences were found ($p<0.05$).

CHAPTER 7

General discussion, conclusions and future directions

7.1 General Discussion

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by an alpha-herpesvirus, Marek's disease virus (MDV) (1, 2). In the absence of control methods, MD can be devastating for the poultry industry (3). MD vaccination has successfully controlled the disease since the first vaccines were introduced in the late 1960's (4-6). However, even when the disease is under control, cost associated to vaccination and to sporadic outbreaks account for over 1 billion USD annually worldwide (7).

MDV induces a variety of syndromes that are divided into two major groups: non neoplastic (transient paralysis, lymphodegenerative syndromes, panophtalmitis, and arteriosclerosis) and neoplastic (lymphoma in viscera, nerves, and skin). In addition, MDV induces immunosuppression (MDV-IS), which is complex and difficult to fit into any of the previous categories. Some of the mechanisms associated with MDV-IS (i.e. early replication of the virus in lymphocytes leading to lymphoid organ atrophy) are not related to tumors. However, there are clear evidence that MD tumor cells can induce immunosuppression (8, 9). Furthermore MDV is able to induce immunosuppression by various other mechanisms (10-14). Because of its complexity, MDV-IS is poorly understood and its relevance in commercial poultry is overlooked. The overall objective of this study was to evaluate if MDV-IS can occur in commercial chickens (bearing maternal antibodies, MAb, against MDV and MD vaccinated), and, if so, to develop a model that could be used to evaluate factors influencing MDV-IS and mechanisms involved.

MDV-IS can be divided into two phases, early immunosuppression (early-MDV-IS) associated with early cytolytic infection of lymphoid organs and late immunosuppression

(late-MDV-IS) that occurs during the establishment of latency and tumor development (15). Early-MDV-IS is controlled by MAb against MDV and vaccination and it is not considered to have any relevance under commercial conditions (16, 17). Our study has focused on the late-MDV-IS.

Our first objective was to develop a model to reproduce late-MDV-IS under laboratory conditions and to demonstrate that late-MDV-IS can occur in commercial meat type chickens bearing MAb against MDV and even vaccinated at day of age against MD. The model we have developed evaluates late-MDV-IS indirectly by assessing the negative effect of MDV on the efficacy of infectious laryngotracheitis (ILT) vaccination (using vaccine adapted to chicken embryo or CEO). Assessment of ILT outcome was done by various criteria: ILT clinical signs, ILT gross lesions, tracheal microscopic lesions, and amount of ILTV gI transcripts in trachea. Because of the consistent pattern from all evaluated criteria, it was concluded that ILT clinical signs and gross lesions are sufficient to evaluate late-MDV-IS in subsequent studies. Using this model, we have shown that late-MDV-IS can occur in commercial chickens with MAb against MDV, vaccinated with MD vaccines at day of age and in total absence of lymphoid organ atrophy or gross tumors. In our study, early infection with vv+MDV 648A strain resulted in marked reduction of CEO efficiency; suggesting that late-MDV-IS might have contributed, at least partially, to the recent outbreaks of ILT in the Southeastern USA. Immunity against ILTV is based in cellular immune responses (18), therefore the model was believed to indirectly evaluated the effect of MDV towards cell mediated immunity. However, in other chapters of this study we have demonstrated that MDV can also affect humoral immune responses (Chapters 4 and 6).

The second objective of this study was to evaluate the efficiency of current vaccination protocols in protecting against late-MDV-IS (Objective 2). The first step was to evaluate if MD vaccines induce immunosuppression on their own. Immunosuppressive ability of MD vaccines has been controversial in the past (19, 20), however, in our study we demonstrated that serotype 1 vaccines (CVI988 and Md5-BACΔMEQ) did not induce late-MDV-IS in our model. The second part of this study was to evaluate if currently used vaccination protocols could protect against late-MDV-IS. Our work demonstrated that none of the currently used MD vaccination protocols, even those that were highly efficacious against MDV-induced tumors, protected against late-MDV-IS induced by vv+ MDV strains 648A and 686. This finding has dual consequences. From the practical perspective, commercial poultry will develop late-MDV-IS, even if they are vaccinated, if they get exposed to vv+MDV strain early in life. In addition, our results force us to reevaluate our thoughts on MDV-IS: late-MDV-IS evaluated in this study does not seem to be related to the development of tumors. In our study there was only one experimental vaccine, Md5-BACΔMEQ that was able to protect against both tumors and late-MDV-IS. Md5-BACΔMEQ is a vaccine originated from vvMDV strain Md5 that lack both copies of the oncogene meq (21, 22). It is considered to be the most protective vaccine against MDV-induced tumors but it induces severe lymphoid organ atrophy in chickens lacking MAb (21, 23, 24). Our study demonstrate that in commercial chickens, Md5-BACΔMeq not only did not induce late-MDV-IS but it was the only vaccine able to protect against it. Mechanisms of protection provided by Md5-BACΔMEQ are still unknown, however, we had shown that Md5-BACΔMEQ not only has impaired its own ability to reactivate from latency in lymphocytes

but also hampered reactivation of vv+MDV 686 strain in chickens co-infected with both viruses. The effect of deleting meq on impairing reactivation from latency in lymphocytes has been confirmed in Chapter 6 using the recombinant strain 686-BACΔMEQ. Our results suggest that meq-deleted vaccines have different mechanism of protection than conventional vaccines (i.e. CVI988) and might involve blocking reactivation of the oncogenic viruses from lymphocytes (in objective 2, vaccine Md5- BACΔMEQ blocked transcription of meq and expression of mdv1-miR-M4-5p of oncogenic strain 686). Such protection not only affect the development of tumors but also MDV-IS.

In objectives 1 and 2, MDV challenge was conducted using vv+MDV strains (648A and 686). vv+MDVs induce more severe MD even in resistant chickens and break vaccine immunity (25), are more neurovirulent (26), replicate faster (27), and induce more lymphoid organ atrophy in chickens lacking MAb (28). In objective 3, we have evaluated the role of pathotype on late-MDV-IS. Our results demonstrated that only vv+MDV (648A and 686) but not v (GA) or vv (Md5) strains induced late-MDV-IS. In addition to the ability to induce late-MDV-IS, vv+MDV strains differed in the level of expression of viral miRNAs (mdv1-miR-M4-5p and mdv1-miR-M2-3p). The role of those viral miRNA on late-MDV-IS remains unknown but it warrants further studies. The effect of virulence was confirmed in objective 5 using vv+MDV strain 686 and its molecular clone 686-BAC. Strain 686 has been reported to be more virulent than its molecular clone 686-BAC (29). In our study 686 but not 686-BAC induced late-MDV-IS, thus confirming that only viruses of the highest virulence induce late-MDV-IS. Our results suggest that MDV-IS is one of the last acquired features of MDV and it is likely involved in increased virulence. If this is the case, as MDV continues evolving, late-

MDV-IS might become a major problem in commercial chickens. Witter (25) showed that 33% of virus isolated in USA between 1993 and 1995 were vv+ strains. Recently, a study conducted in Poland showed that 30% of field isolates belongs to the vv+MDV (30). Our results in objective 2 demonstrated that current MD vaccination does not protect against late-MDV-IS induced by vv+MDV. Therefore, it is likely that a percentage of commercial chickens is suffering of late-MDV-IS. Situation could worsen in the future if the virus become more immunosuppressive. Further studies on the role of MDV-IS on increased MDV virulence are warranted.

Results from previous objectives suggested that late-MDV-IS evaluated in this model might not be related to tumors as protection against tumors did not prevent late-MDV-IS (objective 2). Furthermore, v and vvMDV were able to induce tumors but they did not induce late-MDV-IS (objective 3). In objective 4, we wanted to confirm those findings by two different approaches. First, we wanted to evaluate the role of the oncoprotein meq on late-MDV-IS. Then, we conducted a retrospective study using data from all previous experiment to see if development of tumors was correlated to late-MDV-IS. None of the molecular clones used in the study (Md5-BAC or 686-BAC) induced late-MDV-IS in our model and therefore it was not possible to evaluate the role of meq. However, further studies conducted in objective 5 using viruses derived from vv+MDV strain 686 (686, 686-BAC, and 686-BACΔMEQ) showed that deletion of meq abrogated the negative effects of 686-BAC on humoral immune responses and expression of MHC-I. Meq is a viral oncogene associated with tumor development (22, 31, 32).The role of meq on humoral immunosuppression has been previous reported (33). Our results suggest that meq might be related to some aspects of

the immunosuppression although presence of meq in 686-BAC, Md5-BAC, and Md5 was not sufficient to induce late-MDV-IS in our model. Furthermore, tumors and late-MDV-IS were not correlated in a retrospective study. Our results suggest that late-MDV-IS evaluated in this model and tumors are not related but deletion of meq might reverse some of the negative effect of MDV on the immune responses; probably due to the effect on reactivation of the virus from latency.

Our last objective (Objective 5) evaluates further the pathogenesis of late-MDV-IS. We have used three strains derived from vv+MDV 686 (686, 686-BAC and 686-BACΔMEQ) to evaluate immunosuppressive abilities (late-MDV-IS evaluated in our model and humoral immune responses against ILTV), development of tumors (evaluate by gross inspection and by real time PCR), viral transcripts (ICP4, pp38, meq, and gB), expression of viral miRNA (mdv1-miR-M4-5p and mdv1-miR-M2-3p), and expression of MHC-I in $CD45^+$ splenocytes. Our results showed a clear gradation of virulence and immunosuppressive abilities among the three MDV strains evaluated. The most virulent strain (686) was highly immunosuppressive in the late-MDV-IS model, reduced greatly the humoral immune responses against ILTV, and markedly reduced the percentage of $CD45^+$, MHC-I^+, and $CD45^+$-MHC-I^+ in the spleen. Furthermore, it induces the highest frequency of tumors and had the highest level of MDV transcripts and expression of viral miRNA. In the middle, it was the molecular clone 686-BAC that has been previously reported to be of less virulence than 686 (29). In our study, 686-BAC still reduced expression of antibodies against ILTV to some degree and the percentage of MHC-I^+ cells in the spleen but it did not induced late-MDV-IS in the ILT model. Furthermore it induced tumors and had high MDV

transcripts and high expression of miRNA, albeit at lower level than strain 686. At the bottom of the spectrum was strain 686-BACΔMEQ that did not induce any immunosuppression or tumors, had no effect on MHC-I expression in splenocytes, and have no, or minimal, viral antigen transcription and/or expression of viral miRNAs. Our results confirm that late-MDV-IS evaluated in the ILT model is highly associated with virulence and only the most virulent strains can induce it. Because the ILT model evaluates clinical immunosuppression, we have hypothesized that this model establishes a threshold that only the most virulent strains can overpass. Viruses of lesser virulence (i.e. 686-BAC and possible strains of other pathotypes) can induce some level of immunosuppression (i.e. reduced the percentage of MHC-I^+ cells in spleen and reduced antibody responses) but do not overpass the threshold level of our model. This feature might be useful for further characterization of newly emerging MDVs. Results of objective 5 again emphasized the role of meq in reactivation from latency. As in objective 2, results of this study showed that deletion of meq resulted in blocking reactivation from latency in lymphocytes. This mechanism need further evaluation because it might be the key for future methods of control of both tumors and MDV-IS. Finally, downregulation of MHC-I by MDV has been reported previously (10, 13, 34) and it is a common strategy for herpesvirus to avoid the immune system (35-40). In our study, both 686 and 686-BAC decreased the percentage of MHC-I+ cells in spleen, and 686 decreased also the percentage of $CD45^+MHC\text{-}I^+$. However, since the percentage of $CD45^+$ cells was also reduced it is unknown if 686 down-regulated MHC-I and further studies are warranted. In previous work, it was reported that vv+ but not vMDV were able to down-regulate MHC-I in the brain (13). Our results suggest that virulence might have an effect on

the ability of the virus to down-regulate MHC-I expression and it is possible that this mechanism is involved, at least partially, on the late-MDV-IS evaluated in this study. Further studies are warranted to further evaluate the mechanisms by which MDV can down-regulate MCH-I expression and how MDV virulence affect such ability.

7.2 Conclusions and future directions

The results of this study has expanded our knowledge on MDV-IS, in particular on the late-MDV-IS that is unrelated to lymphoid organ atrophy and tumors. From the practical perspective, our results have major implications: (1) it can occur in commercial poultry and it is most likely occurring whenever chickens get exposed early with a vv+MDV; (2) it affects both cellular and humoral immune responses and can completely abrogate the efficiency of vaccination programs (i.e. ILT); (3) it is not protected by current MD vaccination protocols even by those that are very efficacious against tumors; (4) it appears in the absence of lymphoid organ atrophy and/or tumors, hence the difficulty to diagnose it in the field.

In addition, our studies have a major impact in our understanding on MD pathogenesis and MDV evolution: (1) late-MDV-IS evaluated in the ILT model is unrelated to tumors; (2) it is a feature of the most virulent MDVs and gets easily attenuated, therefore it might be involve in the evolution of MDV and it could be used to further characterize newly emerging strains; (3) Md5-BACΔMEQ was the only vaccine able to protect against tumors and MDV-IS; (4) the ability to regulate MHC-I expression as well as the expression of viral miRNA (mdv1-miR-M4-5p and mdv1-miR-M2-3p) seems to be affected by the MDV virulence, however their role on late-MDV-IS remains unknown and deserve further studies.

Our studies open new avenues for the study of MDV-IS and evolution of MDV. In particular, the role of meq on reactivation from latency and how deletion of meq block such reactivation and subsequently the development of tumors and late-MDV-IS; the role of MDV-IS on the evolution of MDV; the mechanism by which MDV down-regulate MHC-I and to which extent MDV virulence plays a role on the severity of MHC-I down-regulation; and the role of viral miRNA on late-MV-IS are questions that warrant further studies. From the practical point of view it is imperative to find proper methods for the diagnosis of late-MDV-IS and to further evaluate vaccine Md5-BACΔMEQ as it is the only method currently known for the control of late-MDV-IS.

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